REMARKS

This Response to Office Action is submitted together with a Petition to Revive an Unavoidably Abandoned Application, made necessary by the fact that Applicant did not receive the final Office Action, which was mailed January 3, 2002. Briefly, Applicant's undersigned attorney made a status inquiry with the United States Patent & Trademark Office (USPTO) to ascertain why Applicant had not received an Office Action in connection with the above-referenced application, after the departure of Examiner Jill Martin from the USPTO. This status inquiry was prompted by an assessment that an Office Action was overdue. As a result of this status inquiry, Applicant's undersigned attorney learned that a mix-up of some kind had resulted in Applicant never receiving the final Office Action that was issued on January 3, 2002.

On November 7, 2002, Applicant's undersigned attorney received a voicemail message from Supervisory Examiner Deborah Reynolds that the above-captioned application had been assigned to Examiner Woitach. The undersigned immediately contacted Examiner Woitach who told him that a final Office Action had been issued in the above-referenced application on January 3, 2002, and that no response had been received by the USPTO. Applicant's undersigned attorney informed Examiner Woitach that Applicant had never received the Office Action, which as of July 3, 2002, had technically caused the above-captioned application to become abandoned.

Examiner Woitach told Applicant's undersigned attorney that a formal Petition to Revive an Application Unavoidably Abandoned must be filed, which Applicant duly submits herewith.

At Applicant's request, Examiner Woitach kindly transmitted a copy of the Office Action by facsimile, for which Applicant thanks the Examiner.

The Pending Claims:

Before entry of the foregoing amendments, Claims 133-195 are pending in this application. Claims 133-143 and 149-156 are directed to a method of obtaining a selectable transgenic stem cell of a non-human mammal. Claims 144-145, 157-160, and 165-168 are drawn to a selectable transgenic stem cell, and Claims 146, 161, 169 relate to a transgenic non-human mammal comprising the stem cell. Claim 162 relates to a male gamete obtained by the method. Claims 147, 163 and 170 relate to semen of a non-human mammal comprising a male gamete obtained by the method. Claims 148, 164 and 171 relate to a method of producing a non-human transgenic mammalian line. Claims 172 and 174-178 are directed to a transgenic non-human mammalian cell containing a nucleic acid construct, and Claims 173 and 179-180 relate to a transgenic non-human mammal comprising the cell. Claims 181-190 relate to a

method of obtaining a selectable transgenic stem cell of a mouse. Claims 191-192 are drawn to a selectable transgenic stem cell obtained by the method, and Claims 193 relate to a transgenic mouse comprising the stem cell. Claim 194 relates to semen of a male mouse comprising a male gamete obtained by the method. Claim 195 relates to a method of producing a transgenic murine line.

The Office Action and Applicant's Response and Amendment

The Office Action stated that the Examiner of record and art unit had changed. The Examiner of record is now Joseph T. Woitach and the group art unit is now 1632.

The Office Action acknowledged that Applicants' amendment filed October 3, 2001 (mailed September 24, 2001), paper number 16, had been received and entered. In addition, Applicants' supplemental amendment filed October 19, 2001 (mailed October 15, 2001), paper number 17, had been received and entered.

The Office Action acknowledged that the Declaration of Dr. Carol W. Readhead under 37 CFR § 1.132 filed October 3, 2001 (mailed September 24, 2001), Exhibit A attached to paper number 16, had been received and entered.

Claims 136, 172 and 184 were objected to due to the presence of editing notations still present in the claims. The Examiner stated that for the sake of compact prosecution, the claims will be read with the intended edits made, but required appropriate correction.

Accordingly, Applicant has deleted the editing notations from Claims 136, 172, and 184. In addition, Applicant has further amended Claims 136, 149, 172, and 184, for greater clarity, with respect to the operative derivatives of SEQ ID NO:2. Periods are removed from the sequence identifier "SEQ ID NO:2", so that the claims will have only one period each. The recitation of ". . . wherein the derivative does not comprise an operative translational start site at nucleotide positions 1425-1427 of SEQ ID NO:2, and wherein the derivative comprises a Sp1 binding site between nucleotide positions 1188-1262 of SEQ ID NO:2" is supported in the specification as originally filed, for example, at page 20, line 26 through page 22, line 16; page 47, line 1 through page 48, line 8; and in Figures 3 and 6. In particular, nucleotide positions 1425-1427 of SEQ ID NO:2 correspond to nucleotide positions +127 through +129 of the human cyclin A1 promoter, as described at page 21, line 7. Nucleotide positions 1188-1262 of SEQ ID NO:2 correspond to positions -112 through –37, as described at page 21, line 8; and in Figure 3.

The amendment at page 1, line 4 or 5, is to update the continuing data concerning the issuance of U.S. Patent No. 6,316,692, on November 13, 2001, which was noted by the Examiner in the Office Action.

The amendment at page 10, lines 28-29, deleting the phrase ". . . by preventing the methylation of promoter DNA" is to correct an unintentional oversimplification as to the mechanism by which insulator elements are thought to function in protecting a stably integrated gene from chromatin-mediated repression. For example, Burgess-Buesse *et al.* states that mechanism by which insulators provide protection against position effects involves properties that appear to be associated with control of *both* histone acetylation and methylation. **(Exhibit F**: Burgess-Buesse, B. *et al., The insulation of genes from external enhancers and silencing chromatin*, Proc Natl Acad Sci USA 99 Suppl 4:16433-7 [2002], Abstract provided).

In a related division, it was pointed out to Applicant that Figures 2 and 3 contain sequence listings that were not included in Applicant's computer readable form sequence listing. Applicant has submitted herewith a revised computer readable form sequence listing and paper copy.

The amendments at page 12, lines 5 and 6 are to insert sequence designations SEQ ID NO:35 and SEQ ID NO:36, which appear in Figures 2 and 3, respectively. A revised computer readable form sequence listing and paper copy are submitted herewith, which include SEQ ID NO:35 and SEQ ID NO:36.

The amendment at page 17, line 23, is to correct an inadvertent error associating Moloney murine leukemia virus with lentiviruses, but which is actually a member of the retroviral genus *Gammaretrovirus*, and not of the retroviral genus *Lentivirus*, as mistakenly stated originally.

The amendment at page 23, line 15, and at lines 17-19, deleting the independent clause ". . . methylation will be substantially prevented at CG dinucleotide sites within the CpG island of the cyclin A1 promoter sequence and thus expression of the reporter gene occurs within stem cell types other than germ cells" is to correct an unintentional oversimplification as to the mechanism by which insulator elements are thought to function in protecting a stably integrated gene from chromatin-mediated repression. For example, Burgess-Buesse *et al.* states that mechanism by which insulators provide protection against position effects involves properties that appear to be associated with control of *both* histone acetylation and methylation. **(Exhibit**

F: Burgess-Buesse, B. *et al., The insulation of genes from external enhancers and silencing chromatin*, Proc Natl Acad Sci USA 99 Suppl 4:16433-7 [2002], Abstract provided).

The amendment at page 45, line 8 is to insert SEQ ID NOS. A revised computer readable form sequence listing and paper copy are submitted herewith, which include SEQ ID NO:33 and SEQ ID NO:34. Deletion of the quotation marks around the sequences in the text are for greater clarity.

The amendments submitted herein contain no new matter.

The Examiner withdrew the provisional rejection of under the judicially created doctrine of obviousness-type double patenting as being unpatentable over copending Application No. 09/191,920 in view of Applicants having filed a Terminal Disclaimer October 3, 2001, paper number 15, which has obviated the rejection.

The Examiner stated that Claims 133-195 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 124-139 and 157-159 of co-pending Application No. 09/272,443. Applicant had indicated in Paper No. 16 (at page 17) that upon the finding of allowable subject matter in 09/272,443, "Applicant will *consider* executing a terminal disclaimer to the allowable subject matter in the present application." The Examiner stated that the filing of a terminal disclaimer cannot be held in abeyance, and that absent the filing of a terminal disclaimer, the rejection is maintained.

Applicant asserts that the provisional rejection for obviousness type double patenting is negated, because a "cyclin A1 promoter" sequence is recited as a limitation in all independent claims of the above-referenced application. The cyclin A1 promoter sequence was unknown and not suggested by 09/272,443, minus the disclosures of the above-captioned application. Regardless, Applicant submits herewith a Terminal Disclaimer with respect to 09/272,443, thus mooting the provisional rejection.

The Examiner acknowledged that Applicant has "provided the evidence and necessary guidance which provides a nexus between the unpredictability in the art for the transfection of male germ cells and methodology which specifically uses lentivirus and polybrene for the transduction of male germ cells." The Examiner stated that "claims reciting these limitations would be found fully enabled."

Applicant appreciates the Examiner's acknowledgment of enablement, however, with respect to Claims 133-195, Applicant strongly disagrees that the presence of polybrene is an essential limitation. Polybrene was used as a component of the gene delivery mixture in the experiments described in Example 14 and in the Declaration of Dr. Readhead. However, the inclusion of polybrene, or other polycationic agents, such as dioctadecylamidoglycylspermine (DOGS) was known in the art, at the time the above-captioned application was filed, merely for the purpose of enhancing or optimizing transduction efficiency by retroviral vectors, and not as a *necessary* component for transduction (See, e.g., Andreadis, S. and Palsson, B.O., Hum. Gene Ther. 8(3):285-91 [1997], Abstract appended as **Exhibit D**; Movassegh, M. *et al.*, Hum. Gene Ther. 9(2):225-34 [1998], Abstract appended as **Exhibit E**). Thus, retroviral transduction can occur, albeit at a relatively reduced efficiency, even without the presence of polybrene.

Consequently, Applicant asserts that polybrene is an optional component of the recited "transfection mixture" (e.g., Claims 133, 149, 165, 172, and 181), and not an essential limitation with respect to enablement.

Claims 133-195 were subject to other grounds of rejection as detailed hereinbelow, and the Office Action was made final.

I. Rejections based on 35 U.S.C. § 112, first paragraph

Claim 133-195 were rejected under 35 U.S.C. 112, first paragraph, based on the following:

> **. . . Examiner agrees with Applicant that murine models are useful, however the basis of the rejection is not utility, it is the predictability of transgene behavior. The instant specification demonstrates that the specific cyclinAl promoter can be used for expression of a marker gene in the germ cells of a mouse, however there is no nexus that this construct will function in the same manner in other mammals. The art recognises that transgene behavior is unpredictable and absent evidence to the contrary there is no indication that the results presented for the mouse are representative of function in other mammals. The references supplied as Exhibits B-E indicate murine models exist and may be useful, however they do not support that transgene behavior is predictable. For example, there are several art recognized murine models generated for Alzheimer disease (as reviewed by Duff et al. Exhibit E), however among the various models none produce the characteristic plaques seen in humans. Further, Duff et al. indicates that 'many of the mice do not completely replicate the human disease they are intended to model' (in provided abstract). Other exhibits only support the potential utility of future or potential murine models. In addition, it should he pointed out that the methods are directed to isolating stem cells. At the time of filing of the instant application, stem cells from the mouse were the only mammal from which stem cells were successfully isolated. As recognized by one of skill to the art, the specification defines a stem cell as an totipotent cell, however practicing the methods as presently recited would only result in a pluripotent germ cells from a mammal. Because stem cells have been reproducibly isolated from mice, Examiner would concede that the methods are enabled for isolating stem cells from a mouse.**
>
> **The art as exemplified by Sato et al., Lavitrano et al. and Brinster, clearly indicates that the ability to simply transfect/transduce male germ cells was unpredictable at the time of the claimed invention. The specification teaches various methods for the in vivo transfection of germ cells, among them the in vivo transduction of spermatogonia using lentivirus carrying a reporter gene (Example 14), however the results**

presorted in the specification did not clearly indicate that the methodology resulted in the successful or reproducible genetic modification of germ cells. The Declaration of Dr. Carol W. Readhead indicates that following the same method steps as set forth in Example 14, the resulting transduced mice are capable of generating offspring with the transduced marker gene over a course of 20 weeks (summarized in Table 1). These results clearly indicate that methods which use of lentivirus transduction with polybrene results in the successful and reproducible genetic modification of male germ cells. In view of the evidence of record, Examiner would agree that Applicants have provided the evidence and necessary guidance which provides a nexus between the unpredictability in the art for the transfection of male germ cells and methodology which specifically uses lentivirus and polybrene for the transduction of male germ cells. Claims reciting these limitations would be found fully enabled, however, the instant claims encompass broader limitations and are not limited to only this initial transduction of germ cells. Further, the experiments and evidence of recurring support that the cyclin A1 promoter is functional in germ cells, however there is no indication that this promoter is functional in stem cells. Even if the promoter is functional in stem cells, the evidence would suggest then that the promoter is not useful in isolating stem cells through use of the promoter activity because its activity would also be indicative of other cell types.

As to canceled Claims 134, 135, 142, and 145, the rejection is mooted.

Applicant has amended Claims 133, 149, 165, and 181, to recite more narrowly that the transfecting agent "comprises a lipid transfecting agent, a liposome, an adenovirus-enhanced-transferrin-polylysine-DNA complex, or a viral vector, the viral vector being selected from the group consisting of retroviral vectors, mumps vectors, and adenoviral vectors" and to clarify the claims as to relationship of germ cells to the broader category of stem cells, as described in greater detail in Section II of Applicant' response.

With respect to Claims 133, 136-141, 143, 144, and 146-195, Applicant strongly disagrees with the Examiner's basis of rejection, because (1) the specification and the art teach that a stem cell is not necessarily a "totipotent" cell and that germ cells are a subset of stem cells; (2) the specification provides strong empirical evidence that transcription from the human cyclin A1 promoter occurs predictably in a variety of mammalian cells other than mouse cells; (3) the art recognizes that transgenic expression in mice is generally predictive of expression in other mammalian species; and (4) the specification supports the amended claims directed to employing a variety of gene delivery agents other than lentiviral vectors.

(1) <u>The specification and the art recognize that a stem cell is not necessarily a "totipotent" cell and that germ cells are a subset of stem cells.</u>

It is important to understand that *germ cells* are a *subset* of the category "stem cells," and that, contrary to the Examiner's assertion, the specification and the general art recognize that a stem cell need not be a "totipotent" cell (which term "totipotent," could indeed describe a zygotic or embryonic stem cell). (See, e.g., **Exhibit B:** Hogan, B., *Primordial Germ Cells as Stem Cells.* In: *Stem Cell Biology*, Marshak, DR *et al.* [eds], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Chapter 9, pages 189-204 [2001], at second sentence

of page 189). In particular, the specification, as originally filed teaches, e.g., at page 4, lines 18-24:

> A stem cell is an undifferentiated mother cell that is self-renewable over the life of the organism and is multipotent, i.e., capable of generating various committed progenitor cells that can develop into fully mature differentiated cell lines. (citation omitted). All vertebrate tissues arise from stem cells, including hematopoietic stem cells, from which various types of blood cells derive; neural stem cells, from which brain and nerve tissues derive; *and germ cells*, from which male or female gametes derive. (Emphasis added).

Applicant's specification recognizes that "[t]ypes of stem cells for which the method is useful include pluripotent, multipotent, bipotent, or monopotent stem cells, which includes *male or female germ cells* or stem cells related to any tissue of the vertebrate including, but not limited to, spermatogonial, embryonic, osteogenic, hematopoietic, granulopoietic, sympathoadrenal, mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cells," each of which cell lines is less than "totipotent." (Emphasis added; Specification, at page 25, lines 16-22). The specification, as originally filed also describes in detail the developmental pathway (spermatogenesis) of the male germ cell lineage leading to the mature male gamete (e.g., at page 3, lines 7-28).

The specification, as originally filed, provides further enablement for the claimed method, e.g., at page 7, lines 19-26, where it discloses with supporting citations that "cyclin A1 is not expressed in fully differentiated cells of non-embryonic tissues, but can be expressed in a wide variety of stem cells, including male and female germ cells, brain stem cells, hematopoietic progenitor cells, as well as in a majority of myeloid leukemic cells and undifferentiated hematological malignancies." Applicant submitted some of these with an Information Disclosure Statement (July 15, 1999), including: C. Sweeney *et al., A distinct cyclin A is expressed in germ cells in the mouse*, Development 122(1):53-64 [1996]; and R. Yang *et al., Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines*, Cancer Res. 57(5):913-20 [1997]).

The teachings of the specification are consistent with the general knowledge in the art at the time the application was filed, as exemplified in a number of references cited by Applicant in an Information Disclosure Statement, submitted on July 15, 1999: U.S. Patent Nos. 5,591,625; 5,639,618; 5,665,557; and 5,750,376. **U.S. Patent No. 5,639,618** (issued to Gay) states that ". . . stem cells have two main characteristics. First, unlike any other cells, they are capable of dividing and differentiating . . . Second, they are also able to renew themselves . . ." (At column 1, lines 16-31). "The potency of a stem cell is measured by the number of different cell types it can ultimately produce." (At column 1, lines 32-33). The Gay patent goes on to

provide examples of stem cells, none of which is "totipotent": multipotent stem cells, which give rise to two or more different cell types (e.g., hematopoietic stem cells, neuronal stem cells, and neural crest stem cells); bipotent stem cells , which give rise to two cell type (e.g., O-2A progenitors and sympathoadrenal stem cells); and monopotent stem cells, which give rise to only one differentiated cell type (e.g., epidermal stem cell). (At column 1, lines 38-50).

In **U.S. Patent No. 5,750,376** (issued to Weiss *et al.*) "multipotent neural stem cells" are described (See, Abstract and Figure 1), that are capable of proliferating and differentiating by separate pathways into neural cells that include neurons, astrocytes, and oligodendrocytes, in response to suitable physiological conditions (e.g., column 11, line 44 through column 12, line 61). Applicant herewith submits another prefiling date reference, Flax *et al.* appended as **Exhibit A**, describing neural stem cells, which operationally defines them as able to differentiate into cells of all *neural* lineages in multiple regional and developmental contexts; to self-renew; and to populate developing and/or degenerating CNS regions. (**Exhibit A**: Flax, J.D. *et al.*, *Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes*, Nature Biotechnol. 16(11):1033-39 [1998], at page 1033, first paragraph).

U.S. Patent No. 5,665,557 (issued to Murray *et al.*) states that mammalian hematopoietic stem cells ". . . are divided into lymphoid, myeloid, and erythroid lineages," and elaborates on a number of terminally differentiated cell types descending from theses three lines. (See, column 1, lines18-30). Although they are multipotent, hematopoietic stem cells are clearly not "totipotent."

U.S. Patent No. 5,591,625 (issued to Gerson *et al.*) describes mesenchymal stem cells that ". . . can be derived from marrow, periosteum, dermis, and other tissues of mesodermal origin. They are the formative pluripotential blast cells that differentiate into the specific types of connective tissues . . . " (See, column 1, lines 23-30).

By way of further example of the understanding in the art, Hogan, B. [2001] **(Exhibit B)** describes "stem cells" as ". . . a cell population that has the capacity both to self renew and to give rise to *at least one kind* of non-dividing, fully differentiated descendant." (Emphasis added; **Exhibit B**: Hogan [2001], at page 189, last sentence of first paragraph). Thus, a stem cell need not be a "totipotent" cell, contrary to the Examiner's assertion. The Hogan [2001] reference states emphatically, "Germ cells are the precursors of the mature gametes, making their status as stem cells apparently unassailable." **(Exhibit B**: Hogan [2001], at page 189, first sentence).

Another reference, Kiger and Fuller [2001] describe the process of differentiation along the spermatogenic lineage, and state that "due to the amplification divisions, a single daughter cell committed to differentiation eventually gives rise to large numbers of sperm. In

this regard, germ cells are comparable to progenitor cells in the hematopoietic lineage, which maintain a limited proliferative capacity." (citation omitted; **Exhibit C**: Kiger, AA and Fuller, MT, Male Germ-line Stem Cell. In: *Stem Cell Biology*, Marshak, DR *et al.* [eds], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Chapter 8, pages 149-187 [2001], at bridging paragraph of pages 156-157). Thus, the spermatogenic line of stem cells, like the hematopoietic stem cell line can proliferate, under certain physiological conditions, unlike fully differentiated non-stem cells.

Consequently, Applicant's specification and the general knowledge in the art make it clear that the term "stem cell" can refer to a pluripotent, multipotent, bipotent, or monopotent stem cell, and that "stem cell" encompasses a "germ cell." In view of the above and Applicant's amendment of Claims 133, 149, 165, and 181, the Examiner is respectfully requested to withdraw the rejection based on this ground.

(2) The specification provides strong empirical evidence that transcription from the human cyclin A1 promoter occurs predictably in a variety of mammalian cells other than mouse cells.

Contrary to the Examiner's assertion, Applicant's specification provides adequate support and guidance with respect to predictability of application of the claimed invention to non-human mammals other than mice. Applicant's specification, e.g., at page 46, lines 10-13, and at Example 18, describes active expression from the human cyclin A1 promoter, not only in mice and in mouse cells, but in a variety of other mammalian cell lines. The specification states, "Both the -1299 to +144 and the -190 to +144 constructs exhibited promoter activity in a variety of mammalian cell lines including Cos-7 (monkey kidney cell), MCF-7 ([human] breast cancer cell), U937 ([human] myeloid leukemia cell), KCL22 ([human] myeloid leukemia cell), PC3 ([human] prostatic cancer cell), HeLa ([human] cervical cancer cell) and Jurkat ([human] T-cell lymphoma)." (Specification, at page 46, lines 10-13; at Example 20, page 51, lines 1-11; at Example 23, page 56, line 18 through page 57, line 23; Figures 3 and 10). Since Applicant's human cyclin A1 promoter construct is active in rodent and monkey cells, as well as human cells, this is additional strong evidence in the specification that the cyclin A1 promoter is active in other non-human mammals besides mice. Therefore, the Examiner is respectfully requested to withdraw the rejection based on this ground.

(3) The art recognizes that transgenic expression in mice is generally predictive of expression in other mammalian species.

First, contrary to the Examiner's assertion, the Duff reference fails to negate the predictability of expression in cells of non-human mammals other than mice. The Examiner's assertion is simply incorrect that ". . . there are several art recognized murine models generated

for Alzheimer's disease (as reviewed by Duff et al. Exhibit E), however among the various models none produce the characteristic plaques seen in humans." The cited Duff reference (Abstract originally submitted as Exhibit E in Applicant's September 24, 2001 response to Office Action; and appended hereto *in its entirety* as **Exhibit G**: Duff, K., *Transgenic mouse models of Alzheimer's disease: phenotype and mechanisms of pathogenesis*, Biochem. Soc. Symp. 67:195-202 [2001]) teaches to the contrary that there *are* several mouse models of Alzheimer's disease that indeed exhibit such plaques (e.g., at page 195-196, bridging paragraph):

> The first transgenic mouse to develop a robust [Alzheimer's disease] AD-related phenotype was described in 1995 by the Exemplar/Athena Neuroscience group. This line known as PDAPP, overexpresses mutant [amyloid precursor protein] APP at levels high enough to generate sufficient [β-amyloid]Aβ for *extracellular deposits (plaques) to form in relevant regions of the brain.* In 1996, a second line (Tg2576) was created by Karen Hsiao and colleagues, which also *made sufficient amyloid for deposits to form.* In addition, this mouse showed age-related cognitive impairment. Subsequently, *other cDNA mice and mice overexpressing genomic constructs* have also been shown to *form amyloid in old age*. Several groups have created transgenic mice that overexpress mutant [presenilins]PS but these mice do not show amyloid deposition, most likely because they have insufficient levels of the Aβ peptide. (Citations omitted; emphasis added).

Contrary to the Examiner's assertion, the Abstract of the Duff reference teaches, "A range of transgenic mice have been created to model Alzheimer's disease. These include mice expressing human forms of the amyloid precursor protein, the presenelins and, more recently, tau. Several of the models develop features of the disease including *amyloid pathology*, cholinergic deficits, neurodegeneration and cognitive impairment." (Emphasis added; **Exhibit G**: Duff, Abstract, at page 195).

While the Duff reference states that transgenic mouse models of Alzheimer's disease ". . . are, however, still incomplete models as neither tau pathology nor extensive cell loss has been generated in the models created so far. . .", the Duff reference teaches that "[d]espite these shortcomings, they are excellent models of amyloidosis and have been highly informative in advancing our understanding of *in vivo* response to amyloid insult, and the mechanism by which other [Alzheimer's disease] AD-related genes cause the disease." (**Exhibit G**: Duff, at page 199, last paragraph).

Thus, the teachings of Duff illustrate the successes and difficulties of modeling the complex phenotypes of a *multigenic* disease such as Alzheimer's disease, in which the "[g]enetic causes of the disease are heterogeneous and include mutations or variants in *several genes* including those for amyloid precursor protein (APP), the presenelins (PS) and apolipoprotein E." (Emphasis added; **Exhibit G**: Duff, at page 195, middle paragraph; and see, e.g., at page 196, discussing mutant-cross modeling). Under such circumstances of a *multigenic*

disease, it is indeed not surprising that, in mouse models that manipulate only one or another of the implicated genes or even several of them, ". . . many of the mice do not completely replicate the human disease," as the Examiner has noted.

Consequently, the Duff reference fails to negate the predictability of expression in cells of non-human mammals other than mice-- based on Applicant's disclosure of expression in mouse cells-- of a "polynucleotide comprising a transcriptional unit of a human cyclin A1 promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein," in accordance with the claimed invention, e.g., in Claims 133, 165, and 181, or as in amended Claim 149, of a ". . . polynucleotide comprising a transcriptional unit of a cyclin A1 promoter sequence consisting of SEQ ID NO:2, or an operative fragment or derivative thereof. . . said promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein." The claimed invention does not pertain to a multigenic phenotype like Alzheimer's disease, but rather relates to a phenotype involving the transgenic expression of the recited fluorescent or light-emitting protein, which expression can be readily detected by the skilled artisan.

Second, Applicant acknowledges the Examiner's argument that the basis of the rejection is not utility, but rather predictable enablement of transgene expression in accordance with the claimed invention for non-human mammals other than mice. However, Applicant submits that the art recognizes that gene expression in mouse cells *is* generally predictive of gene expression in other mammalian species, and that the utility of mouse models of expression is largely *a consequence of that predictability*. For example, the Wall reference of record (Theriogenology 45:57-68 [1996]) states that testing in the species of interest ". . . is obviously an unsatisfactory, time-consuming, expensive testing option" and that ". . . a reasonable amount of useful information about transgene function *can be* derived from transgenic mouse studies." (Wall at page 62, last paragraph). Useful phenotypic screening means for the effectiveness of a given nucleic acid construct in particular mammals of interest are known and predictable, as taught, for example, by the Wall reference (e.g., at page 62, last eight lines of the last paragraph). The skilled artisan knows that such genetic screening for the desired phenotype is routinely done to detect successful transfections in any single species, since completely uniform gene expression within a population of cells or organisms is practically unachievable in the real biological world. A range of variation of gene expression in individual non-human mammals or cells is the well-recognized rule, which by no means negates the predictability of transgenic expression of a "polynucleotide comprising a transcriptional unit of a human cyclin A1 promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein" in diverse non-human mammalian species in the aggregate, in accordance with the claimed invention.

Moreover, that the art recognizes transgenic expression in mice is generally predictive of expression in other mammalian species is confirmed by Moldin *et al.* (appended hereto *in its entirety* as **Exhibit H**: Moldin, SO *et al.*, *Trans-NIH neuroscience initiatives on mouse phenotyping and mutagenesis*, Mamm. Genome 12(8):575-81 [2001]), which states that "[g]iven the *conservation of cellular and developmental processes from mouse to humans*, an important approach to studying the genetic basis of human disease is to map and characterize genes influencing related biological processes in the mouse." (Emphasis added; at page 575, second column, first complete paragraph). The same conservation of cellular and developmental processes applies to other non-human mammals generally, in accordance with the claimed invention, as Moldin *et al.*, states that "in the post-genomic age, the laboratory mouse undoubtedly will play a pivotal role in understanding the function of *mammalian* genes." (Emphasis added; at page 575, first column, second sentence).

Applicant further submits that the National Institutes of Health recognizes that gene expression in mice is predictive of expression in other mammalian species, as evidenced by its investment in a mouse genetics research program in 1999, which it pursued *based on recommendations of the scientific community* made in the spring of 1998, before the filing date of the above-captioned application. (See, **Exhibit H**: Moldin, SO *et al.*, lines 14-18 of Abstract, at page 575; and at page 576, first column, first two full paragraphs).

In view of the above, it is clear that the art recognizes that transgenic expression in mice is generally predictive of expression in other mammalian species. Therefore, the Examiner is respectfully requested to withdraw the rejection based on this ground.

(4) The specification supports claims directed to employing a variety of gene delivery agents other than lentiviral vectors.

Applicant has amended Claims 133, 149, 165, and 181, to recite more narrowly that the transfecting agent "comprises a lipid transfecting agent, a liposome, an adenovirus-enhanced-transferrin-polylysine-DNA complex, or a viral vector, the viral vector being selected from the group consisting of retroviral vectors, mumps vectors, and adenoviral vectors." Support is found in Applicant's specification, as originally filed, e.g., at page 16, line 29 through page 18, line 1; and especially in the working examples related to lipid transfecting agents, liposomes, and adenovirus-enhanced-transferrin-polylysine-DNA complexes, e.g., at page 32, line 24 through page 38, line 29. The specification teaches that the mumps virus is particularly

suited because of its affinity for immature sperm cells including spermatogonia. (E.g., at page 17, lines 28-29).

In addition, the Declaration of Dr. Readhead under 37 C.F.R. § 1.132 (Exhibit A appended to Response to Office Action mailed by Applicant September 24, 2001; paragraph 5) further demonstrates enablement particularly for a transfecting agent comprising a *retroviral vector*, including, but contrary to the Examiner's assertion, not limited to a lentiviral vector. In particular, Dr. Readhead's declaration discloses the use of an HIV-derived (retroviral genus *Lentivirus*) retroviral vector containing exogenous genetic material, packaged in a broad host-range pseudotyped *retroviral* envelope, i.e., (VSV-G)-Moloney Murine Leukemia Virus (retroviral genus *Gammaretrovirus*; also described in the specification, as originally filed, e.g., at page 17, lines 9-10).

Consequently, Applicant's specification, together with the knowledge in the art at the time the application was filed, indeed provides adequate guidance to the skilled artisan for practicing the method in accordance with amended Claims 133, 149, 165, and 181, and claims dependent directly or indirectly therefrom.

Therefore, the Examiner is respectfully requested to withdraw the rejection under 35 U.S.C. 112, first paragraph.

II. Rejections based on 35 U.S.C. § 112, second paragraph

Claims 133-195 were rejected under 35 U.S.C. 112, second paragraph. The Examiner stated:

> **. . . Claims 133, 149, 165 and dependent claims are unclear and confusing in the recitation of 'a selectable transgenic stem cell' because what is selected or selectable about the stem cell is not clearly defined. Further, the claim indicates that a selectable marker is expressed in a germ cell, however there is not connection between the germ cell and a stem cell.**
>
> **. . . Claim 141 is unclear because it is dependent on claim 133, a method for isolating stem cells, though it is not clear how the development of a gamete is related to isolating a transgenic stem cell.**
>
> **. . . Claim 158 is confusing because a stem cell is totipotent, not bipotent or menopotent. Further, absent somehow directly assaying the resulting cells, it is unclear how one would identify the cell is a pluri-, multi-, bi- or mono-potent cell.**
>
> **. . . Claims 167 and 176 are confusing because a stem cell is totipotent not those recited in the claim. Further, if the promoter carp be used to isolate these cells, it is unclear haw the promoter would he effective in any other method for any one specific cell type.**

Applicant has amended Claims 133, 149, and 165 for greater clarity with respect to the relationship of germ cells to the broader genus of "stem cells," as will be described with particularity below. But first, it is important to understand that *germ cells* are a *subset* of the

category "stem cells," and that, contrary to the Examiner's assertion, the specification and the general art recognize that a stem cell need not be a "totipotent" cell (which term "totipotent," could indeed describe a zygotic or embryonic stem cell). (See, e.g., **Exhibit B:** Hogan, B., *Primordial Germ Cells as Stem Cells.* In: *Stem Cell Biology*, Marshak, DR *et al.* [eds], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Chapter 9, pages 189-204 [2001], at second sentence of page 189). In particular, the specification, as originally filed teaches, e.g., at page 4, lines 18-24:

> A stem cell is an undifferentiated mother cell that is self-renewable over the life of the organism and is multipotent, i.e., capable of generating various committed progenitor cells that can develop into fully mature differentiated cell lines. (citation omitted). All vertebrate tissues arise from stem cells, including hematopoietic stem cells, from which various types of blood cells derive; neural stem cells, from which brain and nerve tissues derive; *and germ cells*, from which male or female gametes derive. (Emphasis added).

Applicant's specification recognizes that "[t]ypes of stem cells for which the method is useful include pluripotent, multipotent, bipotent, or monopotent stem cells, which includes *male or female germ cells* or stem cells related to any tissue of the vertebrate including, but not limited to, spermatogonial, embryonic, osteogenic, hematopoietic, granulopoietic, sympathoadrenal, mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cells," each of which cell lines is less than "totipotent." (Emphasis added; Specification, at page 25, lines 16-22). The specification, as originally filed also describes in detail the developmental pathway (spermatogenesis) of the male germ cell lineage leading to the mature male gamete (e.g., at page 3, lines 7-28).

The specification, as originally filed, provides further enablement for the claimed method, e.g., at page 7, lines 19-26, where it discloses with supporting citations that "cyclin A1 is not expressed in fully differentiated cells of non-embryonic tissues, but can be expressed in a wide variety of stem cells, including male and female germ cells, brain stem cells, hematopoietic progenitor cells, as well as in a majority of myeloid leukemic cells and undifferentiated hematological malignancies." Applicant submitted some of these with an Information Disclosure Statement (July 15, 1999), including: C. Sweeney *et al.*, *A distinct cyclin A is expressed in germ cells in the mouse*, Development 122(1):53-64 [1996]; and R. Yang *et al.*, *Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines*, Cancer Res. 57(5):913-20 [1997]).

The teachings of the specification are consistent with the general knowledge in the art at the time the application was filed, as exemplified in a number of references cited by

Applicant in an Information Disclosure Statement, submitted on July 15, 1999: U.S. Patent Nos. 5,591,625; 5,639,618; 5,665,557; and 5,750,376. **U.S. Patent No. 5,639,618** (issued to Gay) states that ". . . stem cells have two main characteristics. First, unlike any other cells, they are capable of dividing and differentiating . . . Second, they are also able to renew themselves . . ." (At column 1, lines 16-31). "The potency of a stem cell is measured by the number of different cell types it can ultimately produce." (At column 1, lines 32-33). The Gay patent goes on to provide examples of stem cells, none of which is "totipotent": multipotent stem cells, which give rise to two or more different cell types (e.g., hematopoietic stem cells, neuronal stem cells, and neural crest stem cells); bipotent stem cells , which give rise to two cell type (e.g., O-2A progenitors and sympathoadrenal stem cells); and monopotent stem cells, which give rise to only one differentiated cell type (e.g., epidermal stem cell). (At column 1, lines 38-50).

In **U.S. Patent No. 5,750,376** (issued to Weiss *et al.*) "multipotent neural stem cells" are described (See, Abstract and Figure 1), that are capable of proliferating and differentiating by separate pathways into neural cells that include neurons, astrocytes, and oligodendrocytes, in response to suitable physiological conditions (e.g., column 11, line 44 through column 12, line 61). Applicant herewith submits another prefiling date reference, appended as **Exhibit A**, describing neural stem cells, which operationally defines them as able to differentiate into cells of all *neural* lineages in multiple regional and developmental contexts; to self-renew; and to populate developing and/or degenerating central nervous system (CNS) regions. (**Exhibit A**: Flax, J.D. *et al.*, *Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes*, Nature Biotechnol. 16(11):1033-39 [1998], at page 1033, first paragraph).

U.S. Patent No. 5,665,557 (issued to Murray *et al.*) states that mammalian hematopoietic stem cells ". . . are divided into lymphoid, myeloid, and erythroid lineages," and elaborates on a number of terminally differentiated cell types descending from theses three lines. (See, column 1, lines18-30). Although they are multipotent, hematopoietic stem cells, as an example, are clearly not "totipotent."

U.S. Patent No. 5,591,625 (issued to Gerson *et al.*) describes mesenchymal stem cells that ". . . can be derived from marrow, periosteum, dermis, and other tissues of mesodermal origin. They are the formative pluripotential blast cells that differentiate into the specific types of connective tissues . . . " (See, column 1, lines 23-30). Thus, mesenchymal stem cells are another example that stem cells need not be "totipotent," contrary to the Examiner's assertion.

By way of further example of the understanding in the art, Hogan, B. [2001] (**Exhibit B**) describes "stem cells" as ". . . a cell population that has the capacity both to self renew and to give rise to *at least one kind* of non-dividing, fully differentiated descendant." (Emphasis added; **Exhibit B**: Hogan [2001], at page 189, last sentence of first paragraph).

Thus, a stem cell need not be a "totipotent" cell, contrary to the Examiner's assertion. The Hogan [2001] reference states emphatically, "Germ cells are the precursors of the mature gametes, making their status as stem cells apparently unassailable." (**Exhibit B**: Hogan [2001], at page 189, first sentence).

Another reference, Kiger and Fuller [2001] describe the process of differentiation along the spermatogenic lineage, and state that "due to the amplification divisions, a single daughter cell committed to differentiation eventually gives rise to large numbers of sperm. In this regard, germ cells are comparable to progenitor cells in the hematopoietic lineage, which maintain a limited proliferative capacity." (citation omitted; **Exhibit C**: Kiger, AA and Fuller, MT, Male Germ-line Stem Cell. In: *Stem Cell Biology*, Marshak, DR *et al.* [eds], Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Chapter 8, pages 149-187 [2001], at bridging paragraph of pages 156-157). Thus, the spermatogenic line of stem cells, like the hematopoietic stem cell line can proliferate, under certain physiological conditions, unlike fully differentiated non-stem cells.

Consequently, Applicant's specification and the general knowledge in the art make it clear that the term "stem cell" can refer to a pluripotent, multipotent, bipotent, or monopotent stem cell, and that "stem cell" encompasses a "germ cell."

Nevertheless, Applicant *has amended* Claims 133, 149, and 165 for greater clarity with respect to the relationship of germ cells to the broader genus of "stem cells."

In particular, the preamble and final paragraph of Claim 133 have been amended to recite:

> A method of obtaining a transgenic stem cell of a non-human mammal, *said transgenic stem cell being a transgenic germ cell,* . . .
> . . . incorporating said polynucleotide into the genome of said germ cell, whereby a transgenic germ cell is obtained that expresses said fluorescent or light-emitting protein, by the detection of which the transgenic germ cell can be isolated or selected from a population of non-transgenic germ cells.

The last two paragraphs of Claim 149 have been amended for greater clarity to recite:

> . . . allowing said male germ cell to develop into a transgenic male gamete; and
> breeding said male non-human mammal with a female of its species, wherein the *transgenic male gamete fertilizes an ovum*, to obtain a transgenic progeny that comprises at least one transgenic stem cell, the transgenic stem cell being selected from the group consisting of germ cells and somatic stem cells, and the transgenic stem cell expresses said fluorescent or light-emitting protein, whereby the transgenic stem cell can be isolated or selected from a non-stem cell of the transgenic progeny by detecting light emissions from said fluorescent or light-emitting protein.

The last two paragraphs of Claim 165 have been amended to recite:

> . . . causing said polynucleotide to be taken up by, and released into said male germ cell, *wherein said polynucleotide is incorporated into the genome of said germ cell*; and
>
> fertilizing an ovum with said transfected male germ cell such that a transgenic progeny expressing said fluorescent or light-emitting protein in at least one of its stem cells is obtained, said at least one of its stem cells being a transgenic stem cell selected from the group consisting of germ cells and somatic stem cells.

Finally, as to the word "selectable", the recitation of which has been deleted from all claims for the sake of greater clarity, Applicant's specification clarifies what is intended, *i.e.*, that the transgenic stem cell, including a germ cell, that expresses the fluorescent or light-emitting protein can be distinguished and selected, or isolated, by virtue of detecting that expression, from a population of non-expressing cells with which it is found. The specification, at page 25, lines 10-16, teaches:

> A polynucleotide containing the operatively linked stem cell-specific [human cyclin A1] promoter and reporter gene, is incorporated in to the genome of a transfected male germ cell, or precursor, and can be transmitted to progeny after breeding, where it operates in stem cells of the progeny in vivo, such that in a cell population, taken from a progeny vertebrate's tissue or viewed in situ, *stem cells differentially express the reporter gene compared to non-stem cells. Thus, these stem cells are readily selectable from the population of non-stem cells present in the tissue.* (Emphasis added; see, e.g., at page 10, lines 22-24 re cyclin A1 promoter).

The specification goes on to state that "[t]he spermatogonia or stem cells . . . *may be isolated from a mixed cell population* by a novel method including the utilization of a promoter sequence, which is only active in stem cells, for example the cyclin A1 promoter." (Emphasis added; Specification, at page 10, lines 4-7).

In addition, Applicant has clarified in amended Claims 133, 149, and 181, respectively, that the transgenic stem cell (or germ cell) obtained by the claimed method expresses the fluorescent or light-emitting protein ". . . *by the detection of which* the transgenic germ cell can be isolated or selected *from a population of non-transgenic germ cells*" (amended Claims 133 and 181), or, in amended Claim 149 ". . . *whereby the transgenic stem cell can be isolated or selected from a non-stem cell of the transgenic progeny by detecting light emissions from said fluorescent or light-emitting protein.*"

Therefore, in view of the disclosures of the specification and clarifying claim amendments herein, the Examiner is respectfully requested to withdraw the rejection on this ground.

The Examiner stated further that:

> **. . . Claim 146 seems incomplete because it is directed to a transgenic mammal containing a transgenic stem cell of claim 144, however it is unclear how the stem cell is inserted into said mammal. Further, it is noted that a transgenic animal contains a transgene in its germ cells and its somatic cells.**
>
> **. . . Claim 145 is confusing because a stem cell is not the same as a germ cell. It is unclear what the claim intends to encompass, a stem cell or a germ cell.**

To comport with the antecedent basis provided by amended Claim 133, Applicant has also amended Claim 144 to recite, "A selectable transgenic *germ* cell obtained by the method of Claim 133." Similarly, Claim 146 has been amended to recite, "A transgenic non-human mammal comprising the selectable transgenic *germ* cell of Claim 144."

The rejection as to Claim 145 is mooted by the cancellation of Claim 145, which claim was made redundant by the amendment of Claim 144.

Applicant believes the amendments of Claims 133, 144, and 146 overcome the ground of rejection, which the Examiner is respectfully requested to withdraw.

The Examiner also stated the following:

> **. . . Claims 134, 135, 142 are confusing because it depends on a claim for obtaining transgenic stem cells however it seems directed to generating a transgenic mammal. Claim 134 does not further limit claim 133.**

The rejection is mooted by the cancellation of Claims 134, 135, and 142, the subject matter of which is covered in Claims 149, 150, and 155.

The Examiner further stated:

> **. . . Claims 147 and 148 are vague and unclear because the method is not 100% effective in transfection efficiency, and so it is unclear if the claim is directed to only the sperm containing a transgenic or at low efficiency, if the claim would also encompasses sperm/semen from a wild mammal. Further, given that normal sperm is produced one would expect that the breeding would also produce a normal wild type mammal. More clearly indicating that the transgene is present would obviate the basis of the rejection.**

In response, Applicant has amended Claim 147 and 148, as suggested by the Examiner. In particular, amended Claim 147 now recites, "Semen of a non-human mammal comprising a *transgenic* male gamete obtained by the method of Claim 141." Amended Claim 148 now recites, "A method of producing a transgenic non-human mammalian line having native germ cells *comprising a transgene* . . ."

Applicant believes that the amendments of Claims 147 and 148 obviate the grounds of rejection, which the Examiner is respectfully requested to withdraw.

Finally, the Examiner also stated that:

. . . Claims 137 and 151 are unclear and confusing because insulator elements do not prevent methylation, it is the primary polynucleotide sequence which dictates whether the sequence will be methylated.

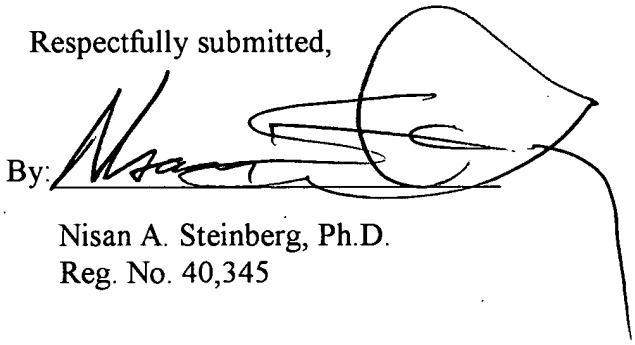
In response, Applicant has overcome the rejection by amending Claims 137 and 151 to delete the whereby clause pertaining to methylation, which is superfluous.

Consequently, Applicant respectfully requests the Examiner to withdraw the rejection of Claims 137 and 151.

CONCLUSION

In view of the above amendments and remarks, it is submitted that this application is now ready for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney at (213) 896-6665.

Respectfully submitted,

By: 

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Version With Markings To Show Changes Made

Brackets to designate deletions are in bold typeface to distinguish them from brackets that are an integral part of the text.

In the Specification:

At page 1, lines 3-5, please delete the paragraph, and insert the following paragraph therefor:

--This application is a continuation-in-part of U.S. Patent Application 09/191,920, filed November 13, 1998, which issued as U.S. Patent No. 6,316,692, on November 13, 2001, and which claims the benefit of U.S. Provisional Application No. 60/065825, filed on November 14, 1997.--.

At page 10, lines 22-30, please delete the paragraph, and insert the following paragraph therefor:

--The present invention also relates to a method of obtaining selectable transgenic stem cells by transfecting a male germ cell with a DNA construct comprising a stem cell-specific promoter, for example, a cyclin A1 promoter, operatively linked to a gene encoding a fluorescent or light-emitting reporter protein. The present invention also relates to selectable transgenic stem cells that have stably integrated the DNA and non-human transgenic vertebrates comprising them. In stem cells other than germ cells, expression of the reporter gene from a cyclin A1 promoter in vivo is facilitated **[**by preventing the methylation of promoter DNA**]** by the use of flanking insulator elements. Alternatively, when transgenic stem cells are grown in vitro, inhibitors of DNA methylation can be added to the culture medium.--.

At page 12, line 5, please delete the paragraph, and insert the following paragraph therefor:

--Figure 2 represents transcriptional start sites in the human cyclin A1 gene (nucleotide positions –70 through +10 are shown; SEQ ID NO:35).--.

At page 12, line 6, please delete the paragraph, and insert the following paragraph therefor:

--Figure 3 represents 5' upstream region of the human cyclin A1 gene (nucleotide positions –1299 through +152 are shown; SEQ ID NO:36).--.

Please delete the paragraph at page 17, line 20 through page 18, line 1, and insert therefor the following paragraph:

--"Virus", as used herein, means any virus, or transfecting fragment thereof, which may facilitate the delivery of the genetic material into male germ cells. Examples of viruses which are suitable for use herein are adenoviruses, adeno-associated viruses, retroviruses such as human immune-deficiency virus, [lentiviruses, such as] Moloney murine leukemia virus and the retrovirus vector derived from Moloney virus called vesicular-stomatitis-virus-glycoprotein (VSV-G)-Moloney murine leukemia virus, mumps virus, and transfecting fragments of any of these viruses, and other viral DNA segments that facilitate the uptake of the desired DNA segment by, and release into, the cytoplasm of germ cells and mixtures thereof. The mumps virus is particularly suited because of its affinity for immature sperm cells including spermatogonia. All of the above viruses may require modification to render them non-pathogenic or less antigenic. Other known vector systems, however, may also be utilized within the confines of the invention.--.

At page 23, lines 12-24, please delete the paragraph, and insert the following paragraph therefor:

--Therefore, for the purposes of obtaining selectable transgenic stem cells in accordance with the present method, silencing of expression from the cyclin A1 promoter in stem cell types other than germ cells is preferably prevented by flanking the promoter sequence and the reporter gene with insulator elements[. F], for example, by including double copies of the 1.2 kb chicken β-globin insulator element 5' to the cyclin A1 promoter sequence and 3' to the reporter protein gene in the present DNA construct.[, methylation will be substantially prevented at CG

dinucleotide sites within the CpG island of the cyclin A1 promoter sequence and thus expression of the reporter gene occurs within stem cell types other than germ cells.] (M.J. Pikaart *et al.*, *Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators*, Genes Dev. 12:2852-62 [1998]; Chung *et al.*, *DNA sequence which acts as a chromatin insulator element to protect expressed genes from cis-acting regulatory sequences in mammalian cells*, U.S. Patent No. 5,610,053).--.

Please delete the paragraph at page 45, lines 5-11, and insert the following paragraph therefor:

--Genomic sequences 1299 bp upstream of the transcription start site were cloned and sequenced. No TATA box was found in proximity to the putative transcriptional start site. The main transcriptional start site is likely to function as an initiator region (Inr) since the sequence ["]CCAGTT["] (SEQ ID NO:33) is very similar to the consensus Inr sequence ["]TCA G/T T T/C["] (SEQ ID NO:34; T.W. Burke and J.T. Kadonaga, Genes & Development 1:3020-31 [1997]). No DPE element was found downstream of the main transcriptional start site. (See *id.*). Several potential binding sites for transcription factors occur within the sequence.--.

In the Claims:

Please cancel Claims 134, 135, 142, 143, and 145, without prejudice, and amend Claims 133, 136, 137, 141, 144, 146-149, 151, 155-170, 172, 173, 175, 181, 182, 184, and 188-195 as follows.

133.(Amended) A method of obtaining a [selectable] transgenic stem cell of a non-human mammal, said transgenic stem cell being a transgenic germ cell, comprising:

injecting into a gonad of a male non-human mammal a transfection mixture comprising at least one transfecting agent that comprises a lipid transfecting agent, a liposome, an adenovirus-enhanced-transferrin-polylysine-DNA complex, or a viral vector, the viral vector being selected from the group consisting of retroviral vectors, mumps vectors, and adenoviral vectors, and at least one polynucleotide comprising a transcriptional unit of a human cyclin A1 promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein, wherein said gonad contains a male germ cell of the non-human mammal, and wherein said germ

cell is selected from the group consisting of spermatogonial stem cells, type B spermatogonia, primary spermatocytes, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, secondary spermatocytes, spermatids, and spermatozoa;

causing said polynucleotide to be taken up by, and released into, said germ cell; and

incorporating said polynucleotide into the genome of said germ cell, whereby a [selectable] transgenic [stem]germ cell is obtained that expresses[ing] said fluorescent or light-emitting protein, by the detection of which [said stem]the transgenic germ cell can be isolated or selected from a population of non-transgenic germ cells[non-stem cell].

134.(Canceled)

135.(Canceled)

136.(Amended) The method of Claim 133, wherein said cyclin A1 promoter sequence comprises SEQ[.] ID[.] NO[.]:2, or an operative fragment [thereof] of SEQ ID NO:2, or an operative derivative of any of these, wherein the derivative does not comprise an operative translational start site at nucleotide positions 1425-1427 of SEQ ID NO:2, and wherein the derivative comprises a Sp1 binding site between nucleotide positions 1188-1262 of SEQ ID NO:2.

137.(Amended) The method of Claim 133, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit[, whereby methylation in vivo of said promoter sequence is substantially prevented].

138.(Reiterated) The method of Claim 137, wherein at least one of said insulator element(s) is a chicken β-globin insulator element.

139.(Reiterated) The method of Claim 133, wherein said fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase or apoaequorin.

140.(Reiterated) The method of Claim 133, wherein said non-human mammal is a non-human primate, a mouse, a rat, a rabbit, a gerbil, a hamster, a canine, a feline, an ovine, a bovine, a swine, a pachyderm, an equine, or a marine mammal.

141.(Amended) The method of Claim 133, wherein [said]the transgenic germ cell develops into a male gamete after said polynucleotide is incorporated into the genome of said germ cell.

142.(Canceled)

143.(Canceled)

144.(Amended) A [selectable] transgenic [stem]germ cell obtained by the method of Claim 133.

145.(Canceled)

146.(Amended) A transgenic non-human mammal comprising the [selectable] transgenic [stem]germ cell of Claim 144.

147.(Amended) Semen of a non-human mammal comprising [a]the transgenic male gamete obtained by the method of Claim 141.

148.(Amended) A method of producing a transgenic non-human mammalian line having native germ cells comprising a transgene, comprising

breeding of the non-human mammal of Claim 146 with a member of the opposite sex of the same species; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light-emitting protein.

149.(Amended) A method of obtaining a [selectable] transgenic stem cell of a non-human mammal, comprising:

injecting into a gonad of a male non-human mammal a transfection mixture comprising at least one transfecting agent that comprises a lipid transfecting agent, a liposome, an adenovirus-enhanced-transferrin-polylysine-DNA complex, or a viral vector, the viral vector being selected from the group consisting of retroviral vectors, mumps vectors, and adenoviral vectors, and at least one polynucleotide comprising a transcriptional unit of a cyclin A1 promoter sequence consisting of SEQ[.] ID[.] NO[.]:2, or an operative fragment or derivative thereof, wherein the derivative does not comprise an operative translational start site at nucleotide positions 1425-1427 of SEQ ID NO:2, and wherein the derivative comprises a Sp1 binding site

between nucleotide positions 1188-1262 of SEQ ID NO:2, said promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein, wherein said gonad contains a male germ cell of the non-human mammal, and wherein said germ cell is selected from the group consisting of spermatogonial stem cells, type B spermatogonia, primary spermatocytes, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, secondary spermatocytes, spermatids, and spermatozoa;

causing said polynucleotide to be taken up by, and released into, said male germ cell;

incorporating said polynucleotide into the genome of said germ cell;

allowing said male germ cell to develop into a transgenic male gamete; and

breeding said male non-human mammal with a female of its species, wherein the transgenic male gamete fertilizes an ovum, to obtain a transgenic progeny that comprises [expressing said fluorescent or light-emitting protein in] at least one [of its] transgenic stem cell[s], the transgenic stem cell being selected from the group consisting of germ cells and somatic stem cells, and the transgenic stem cell expresses said fluorescent or light-emitting protein, whereby [said]the transgenic stem cell can be isolated or selected from a non-stem cell of the transgenic progeny by detecting light emissions from said fluorescent or light-emitting protein.

150.(Reiterated) The method of Claim 149, wherein breeding is by in vitro or in vivo fertilization of an ovum of said female.

151.(Amended) The method of Claim 149, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit[, whereby methylation in vivo of said promoter sequence is substantially prevented].

152.(Reiterated) The method of Claim 151, wherein at least one of said insulator element(s) is a chicken β-globin insulator element.

153.(Reiterated) The method of Claim 149, wherein said fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase or apoaequorin.

154.(Reiterated) The method of Claim 149, wherein said non-human mammal is a non-human primate, a mouse, a rat, a rabbit, a gerbil, a hamster, a canine, a feline, an ovine, a bovine, a swine, a pachyderm, an equine, or a marine mammal.

155.(Amended) The method of Claim 149, further comprising growing [a]the transgenic stem cell of said transgenic progeny in vitro.

156.(Amended) The method of Claim 155, wherein [said]the transgenic stem cell is grown in the presence of an inhibitor of DNA methylation.

157.(Amended) A [selectable] transgenic stem cell obtained by the method of Claim 149.

158.(Amended) The [selectable] transgenic stem cell of Claim 157, wherein said stem cell is a pluripotent, multipotent, bipotent, or monopotent stem cell.

159.(Amended) The [selectable] transgenic stem cell of Claim 157, wherein said stem cell is a spermatogonial, embryonic, osteogenic, hematopoietic, granulopoietic, sympathoadrenal, mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cell.

160.(Amended) The [selectable] transgenic stem cell of Claim 157, wherein said stem cell is a [selectable] transgenic female or a [selectable] transgenic male germ cell.

161.(Amended) A transgenic non-human mammal comprising the transgenic stem cell of Claim 157.

162.(Amended) A transgenic male gamete obtained by the method of Claim 149.

163.(Amended) Semen comprising the transgenic male gamete of Claim 162.

164.(Amended) A method of producing a transgenic non-human mammalian line having native germ cells, comprising

breeding the transgenic non-human mammal of Claim 161 with a member of the opposite sex of the same species; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light-emitting protein.

165.(Amended) A [selectable] transgenic stem cell obtained by:

obtaining a male germ cell from a non-human mammal;

transfecting said male germ cell in vitro with a transfection mixture comprising at least one transfecting agent that comprises a lipid transfecting agent, a liposome, an adenovirus-enhanced-transferrin-polylysine-DNA complex, or a viral vector, the viral vector being selected from the group consisting of retroviral vectors, mumps vectors, and adenoviral vectors, and at least one polynucleotide comprising a transcriptional unit of a human cyclin A1 promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein, wherein said male germ cell is selected from the group consisting of spermatogonial stem cells, type B spermatogonia, primary spermatocytes, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, secondary spermatocytes, spermatids, and spermatozoa;

causing said polynucleotide to be taken up by, and released into said male germ cell, wherein said polynucleotide is incorporated into the genome of said germ cell; and

fertilizing an ovum with said transfected male germ cell such that a transgenic progeny expressing said fluorescent or light-emitting protein in at least one of its stem cells is obtained, said at least one of its stem cells being a transgenic stem cell selected from the group consisting of germ cells and somatic stem cells[stem cell(s) being selectable from non-stem cells by detecting light emissions from said stem cell(s)].

166.(Amended) The [selectable] transgenic stem cell of Claim 165, wherein [said]the transgenic stem cell is a pluripotent, multipotent, bipotent, or monopotent stem cell.

167.(Amended) The [selectable] transgenic stem cell of Claim 165, wherein said stem cell is a spermatogonial, embryonic, osteogenic, hematopoietic, granulopoietic, sympathoadrenal, mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cell.

168.(Amended) The [selectable] transgenic stem cell of Claim 165, wherein [said]the transgenic stem cell is a [selectable] transgenic female germ cell or a [selectable] transgenic male germ cell.

169.(Amended) A transgenic non-human mammal comprising the [selectable] transgenic stem cell of Claim 165.

170.(Amended) Semen comprising the transgenic male germ cell of Claim 168.

171.(Reiterated) A method of producing a transgenic non-human mammalian line having native germ cells, comprising

breeding the non-human mammal of Claim 169 with a member of the opposite sex of the same species; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light-emitting protein.

172.(Amended) A transgenic non-human mammalian cell [containing] comprising a nucleic acid construct, said nucleic acid construct comprising a human cyclin A1 promoter having nucleotide sequence [(]SEQ[.] ID[.] NO[.]:2[)], or an operative fragment of SEQ ID NO:2, or an operative derivative of any of these, wherein the derivative does not comprise an operative translational start site at nucleotide positions 1425-1427 of SEQ ID NO:2, and wherein the derivative comprises a Sp1 binding site between nucleotide positions 1188-1262 of SEQ ID NO:2.

173.(Amended) A transgenic non-human mammal comprising the transgenic non-human mammalian cell of Claim 172.

174.(Reiterated) The transgenic non-human mammalian cell of Claim 172, wherein said cell is a transgenic stem cell.

175.(Amended) The transgenic stem cell of Claim 174, wherein [said]the transgenic stem cell is a pluripotent, multipotent, bipotent, or monopotent stem cell.

176.(Reiterated) The transgenic stem cell of Claim 174, wherein said stem cell is a spermatogonial, hematopoietic, embryonic, osteogenic, granulopoietic, sympathoadrenal, mesenchymal, epidermal, neuronal, neural crest, O-2A progenitor, brain, kidney, pancreatic, liver or cardiac stem cell.

177.(Reiterated) The transgenic stem cell of Claim 174, grown in vitro.

178.(Reiterated) The transgenic stem cell of Claim 177, grown in the presence of an inhibitor of DNA methylation.

179.(Reiterated) A transgenic non-human mammal comprising the transgenic stem cell of Claim 174.

180.(Reiterated) The transgenic non-human mammal of Claim 179, wherein said non-human mammal is a non-human primate, a mouse, a rat, a rabbit, a gerbil, a hamster, a canine, a feline, an ovine, a bovine, a swine, a pachyderm, an equine, or a marine mammal.

181.(Amended) A method of obtaining a [selectable] transgenic stem cell of a mouse, comprising:

injecting into a gonad of a male mouse a transfection mixture comprising at least one transfecting agent that comprises a lipid transfecting agent, a liposome, an adenovirus-enhanced-transferrin-polylysine-DNA complex, or a viral vector, the viral vector being selected from the group consisting of retroviral vectors, mumps vectors, and adenoviral vectors, and at least one polynucleotide comprising a transcriptional unit of a human cyclin A1 promoter sequence operatively linked to a DNA encoding a fluorescent or light-emitting protein, wherein said gonad contains a male germ cell of the mouse, and wherein said germ cell is selected from the group consisting of spermatogonial stem cells, type B spermatogonia, primary spermatocytes, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, secondary spermatocytes, spermatids, and spermatozoa;

causing said polynucleotide to be taken up by, and released into, said germ cell; and

incorporating said polynucleotide into the genome of said germ cell, whereby a [selectable] transgenic stem cell is obtained, the transgenic stem cell being a transgenic germ cell that expresses[ing] said fluorescent or light-emitting protein, by the detection of which [said stem]the transgenic germ cell can be isolated or selected from a population of non-transgenic germ cells[non-stem cell].

182.(Amended) The method of Claim 181, further comprising, after incorporating said polynucleotide into the genome of said germ cell, breeding said male mouse with a female mouse to obtain a transgenic progeny expressing said fluorescent or light-emitting protein in at least one of its stem cells, said stem cell being a transgenic stem cell selected from the group consisting of germ cells and somatic stem cells.

183.(Reiterated) The method of Claim 182, wherein breeding is by in vitro or in vivo fertilization of an ovum of said female mouse.

184.(Amended) The method of Claim 181, wherein said cyclin A1 promoter sequence comprises SEQ[.] ID[.] NO[.]:2, or an operative fragment [thereof] of SEQ ID NO:2, or an operative derivative of any of these, wherein the derivative does not comprise an operative translational start site at nucleotide positions 1425-1427 of SEQ ID NO:2, and wherein the derivative comprises a Sp1 binding site between nucleotide positions 1188-1262 of SEQ ID NO:2.

185.(Reiterated) The method of Claim 181, wherein said polynucleotide further comprises at least one insulator element flanking said transcriptional unit, whereby methylation in vivo of said promoter sequence is substantially prevented.

186.(Reiterated) The method of Claim 185, wherein at least one of said insulator element(s) is a chicken β-globin insulator element.

187.(Reiterated) The method of Claim 181, wherein said fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase or apoaequorin.

188.(Amended) The method of Claim 181, wherein [said]the transgenic germ cell develops into a transgenic male gamete after said polynucleotide is incorporated into the genome of said germ cell.

189.(Amended) The method of Claim 182, further comprising growing [a]the transgenic stem cell of said progeny in vitro.

190.(Amended) The method of Claim 189, wherein [said]the transgenic stem cell of said progeny is grown in the presence of an inhibitor of DNA methylation.

191.(Amended) A [selectable] transgenic stem cell obtained by the method of Claim 182[1].

192.(Amended) The [selectable] transgenic stem cell of Claim 191, wherein [said]the transgenic stem cell is a [selectable] transgenic male germ cell.

193.(Amended) A transgenic mouse comprising the [selectable] transgenic stem cell of Claim 191.

194.(Amended) Semen of a male mouse comprising [a]the transgenic male gamete obtained by the method of Claim 188.

195.(Amended) A method of producing a transgenic murine line having native germ cells, comprising

breeding [of] the transgenic mouse of Claim 193 with a mouse of the opposite sex; and selecting progeny for stem cell-specific expression of a xenogeneic fluorescent or light-emitting protein.

EXHIBIT A

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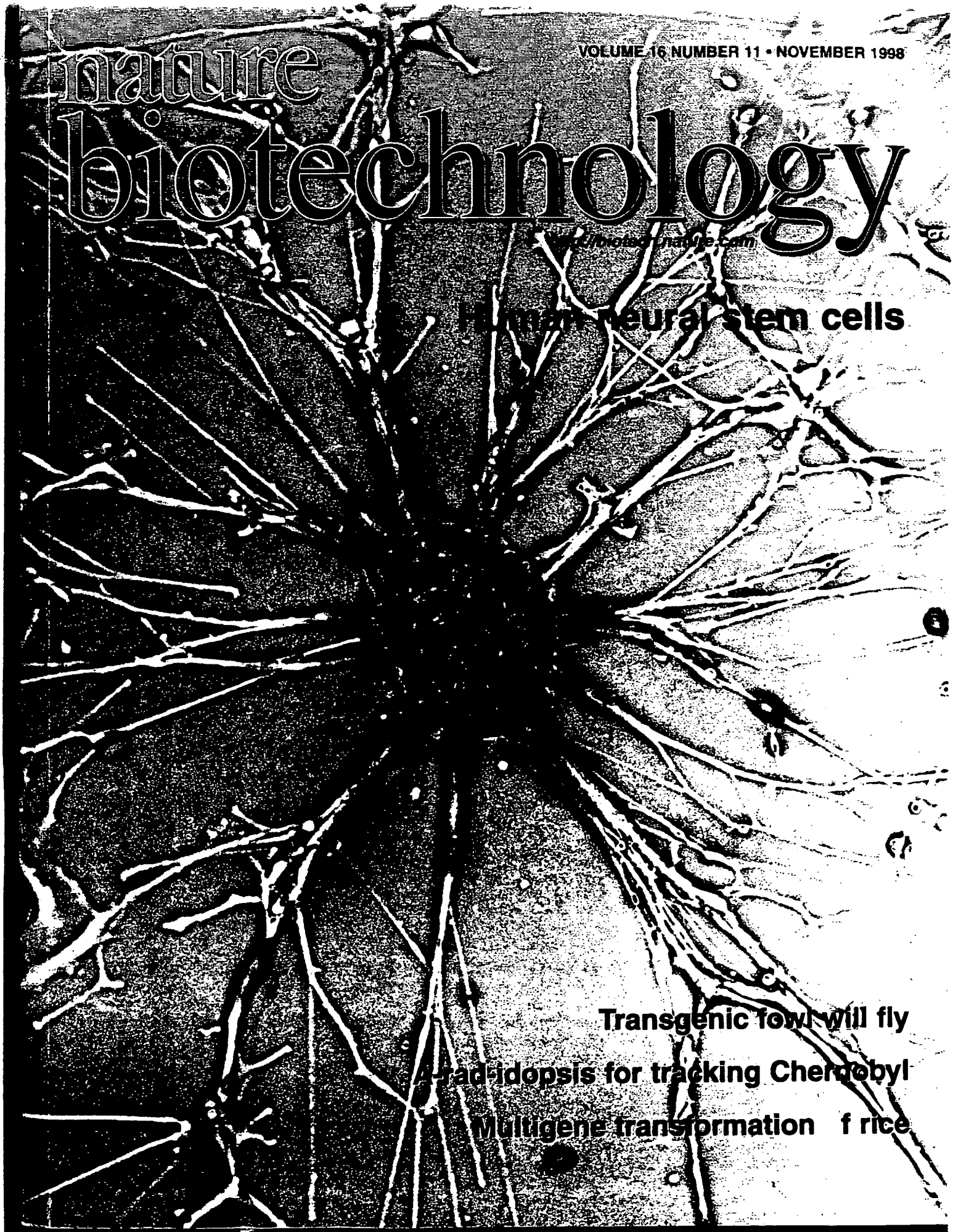

Human neural stem cells

Transgenic fowl will fly

Arabidopsis for tracking Chernobyl

Multigene transformation of rice

Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes

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Stable clones of neural stem cells (NSCs) have been isolated from the human fetal telencephalon. These self-renewing clones give rise to all fundamental neural lineages in vitro. Following transplantation into germinal zones of the newborn mouse brain they participate in aspects of normal development, including migration along established migratory pathways to disseminated central nervous system regions, differentiation into multiple developmentally and regionally appropriate cell types, and nondisruptive interspersion with host progenitors and their progeny. These human NSCs can be genetically engineered and are capable of expressing foreign transgenes in vivo. Supporting their gene therapy potential, secretory products from NSCs can correct a prototypical genetic metabolic defect in neurons and glia in vitro. The human NSCs can also replace specific deficient neuronal populations. Cryopreservable human NSCs may be propagated by both epigenetic and genetic means that are comparably safe and effective. By analogy to rodent NSCs, these observations may allow the development of NSC transplantation for a range of disorders.

Keywords: cell therapy, progenitor cell, gene therapy, Tay-Sachs disease, transplanation, differentiation

Neural stem cells (NSCs) are primordial, uncommitted cells postulated to give rise to the array of more specialized cells of the central nervous system (CNS)[1-4]. They are operationally defined by their ability (1) to differentiate into cells of all neural lineages (i.e., neurons—ideally of multiple subtypes, oligodendroglia, astroglia) in multiple regional and developmental contexts; (2) to self-renew (to give rise to new NSCs with similar potential); and (3) to populate developing and/or degenerating CNS regions. The demonstration of a monoclonal derivation of progeny is obligatory to the definition (i.e., a single cell must possess these attributes). With the earliest recognition that rodent neural cells with stem cell properties, propagated in culture, could be reimplanted into mammalian brain where they could reintegrate appropriately and stably express foreign genes[10-13], gene therapists and neurobiologists began to speculate how such a phenomenon might be harnessed for therapeutic advantage as well as for understanding developmental mechanisms. These, and the studies they spawned (reviewed in refs. 14–16), provided hope that the use of NSCs might circumvent some limitations of presently available graft material[16] and gene transfer vehicles[17] and make feasible a variety of therapeutic strategies.

Neural cells with stem cell properties have been isolated from the embryonic, neonatal, and adult rodent CNS and propagated in vitro by a variety of equally effective and safe means—both epigenetic (with mitogens such as epidermal growth factor [EGF][5] or basic fibroblast growth factor [bFGF][13,18,19] or with membrane substrates[7]) and genetic (with propagating genes[15] such as *v-myc*[10,20] or large T-antigen [*T-Ag*][9]). Maintaining NSCs in a proliferative state in culture does not subvert their ability to respond to normal developmental cues in vivo following transplantation (such as the ability to withdraw from the cell cycle, interact with host cells, and differentiate[1,8]). These extremely plastic cells migrate and differentiate in a temporally and regionally appropriate manner particularly following implantation into germinal zones. Intermingling nondisruptively with endogenous progenitors, responding similarly to local cues for their phenotypic determination, and appropriately differentiating into diverse neuronal and glial types, they participate in normal development along the rodent neuraxis. In addition, they can express foreign genes in vivo[9-16], often in widely disseminated CNS regions[21,22], and are capable of neural cell replacement[4].

The presumption has been that the biology that endows such rodent cells with their therapeutic potential is conserved in the human CNS. If true, then progress toward human applications may be accelerated. We demonstrate the potential of clones of human NSCs to perform these critical functions in vitro and in vivo in a manner analogous to their rodent counterparts.

Results and discussion

Isolation, propagation, and cloning of human NSCs. The isolation, propagation, characterization, cloning, and transplantation of NSCs from the human CNS mirrored strategies used for the murine NSC clone C17.2 (propagated following transduction of a constitutively downregulated *v-myc*[10,21]) and for growth factor-expanded murine NSC clones[1,3]. NSCs—even genetically propagated clones[23]—require molecules like bFGF and/or EGF in serum-free medium to divide[1,3,4]. Therefore, this dual responsiveness was

chosen for both screening and enriching a starting population of stable, dissociated, cultured primary human neural tissue for cells. Cells dissociated from human fetal telencephalon—particularly the ventricular zone, which has been postulated to harbor (in lower mammals) a rich NSC population—were initially grown as a polyclonal population first in serum-supplemented and then in serum-free medium containing bFGF and/or EGF. Cells were transferred between media containing one or the other of the mitogens to select for dual responsiveness. Some populations were then maintained in bFGF alone for subsequent manipulation and cloning; others were used for retrovirally mediated transduction of *v-myc* and subsequent cloning.

To provide an unambiguous molecular tag for assessing the clonal relationships of the cells, as well as to facilitate identification of some cells following transplantation and to assess their capacity to express exogenous genes in vivo, some bFGF-propagated subpopulations were infected with an amphotropic replication-incompetent retroviral vector encoding *lacZ* (and *neo* for selection). Single resistant colonies were initially isolated by limiting dilution. Monoclonality of the cells in a given colony was then confirmed by demonstrating the presence of only one copy of the *lacZ/neo*-encoding retrovirus, with a unique chromosomal insertion site. In clone H1, for example, all *lacZ/neo*-positive cells, had a single, common retroviral integration site indicating that they were derived from a single infected "parent" cell (Fig. 1A).

In rodents, genes (such as *v-myc* and *T-Ag*) that interact with cell cycle regulatory proteins have been used to propagate NSCs[8,9], neural progenitors[25], and neuroblasts[12], resulting in engraftable rodent NSC clones that can be manipulated and have therapeutic potential[14]. Therefore, some of the bFGF-maintained human cell populations, enriched for NSCs, were infected with an amphotropic, replication-incompetent retroviral vector encoding *v-myc* and *neo*[20] yielding multiple colonies. All of the putative clones had only one unique retroviral insertion site, demonstrating their monoclonality (Fig. 1B). Five clones (H6, H9, D10, C2, and E11) were generated and maintained in serum-free medium containing bFGF.

Multipotency and self-renewal in vitro. In uncoated dishes and in serum-free medium supplemented with bFGF, all clones grew in culture as clusters that could be passaged weekly for at least 1 year (Fig. 2A). The cells within these clusters expressed vimentin, a neural progenitor marker[18]. By dissociating these clusters and plating them in serum-containing medium, these clones differentiated spontaneously into neurons and oligodendrocytes (Figs. 2B and C). After 5 days under these differentiating conditions, 90% of the cells in all clones became immunoreactive for the neuronal marker neurofilament (NF; Fig. 2B); 10% expressed CNPase, a marker for oligodendroglia (Fig. 2C). Mature astroglia containing glial fibrillary acidic protein (GFAP) were not initially observed, even after 1 month under these culture conditions. However, GFAP production could be induced by coculture with primary dissociated embryonic murine CNS tissue (Fig. 2D). In addition to cells expressing the variety of differentiated lineage-specific markers (establishing "multipotency"), each clone gave rise to new immature vimentin-positive cells (Fig. 2E), which could, upon subsequent passage, give rise to new cells expressing multiple differentiated neural markers as well as to new vimentin-positive passageable cells (i.e., "self-renewability"). All the clones, whether genetically modified or epigenetically maintained, were similar in vitro.

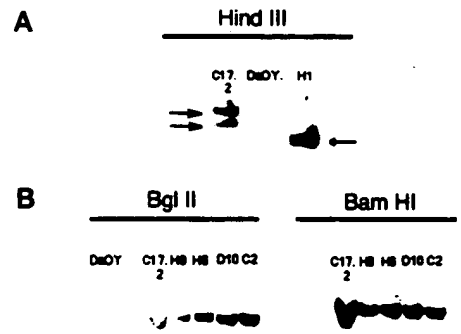

Figure 1. Southern blot analysis of retroviral insertion into human NSC clones. (A) Genomic DNA from clone H1 (propagated in bFGF and transduced with a retrovirus encoding *lacZ* and *neo*) digested with Hind III (cuts once within the provirus) and incubated with a radiolabeled *neo* probe. The murine NSC clone C17.2 contains two integrated proviruses encoding *neo*[13,25]. DaOY is an uninfected human medulloblastoma cell line. (B) Genomic DNA from clones H9, H6, D10, and C2 (propagated in bFGF and/or EGF and infected with a retrovirus encoding *v-myc*) were digested with BglII or BamHI (cuts once within the provirus) and probed for *v-myc*. C17.2 contains one *v-myc*-encoding provirus.

Ability to cross-correct a genetic defect. To assess their potential as vehicles for molecular therapies, we compared the ability of human NSCs to complement a prototypical genetic defect to murine NSCs[26]. The neurogenetic defect chosen was in the α-subunit of β-hexosaminidase, a mutation that leads to hexosaminidase-A deficiency and a failure to metabolize GM_2 ganglioside to GM_3 (Tay-Sachs disease [TSD]). Pathologic GM_2 accumulation in the brain leads to progressive neurodegeneration. The ability of human NSCs to cross-correct was compared with that of two established murine NSC clones: C17.2 and a subclone of C17.2 (C17.2H) engineered via retroviral transduction of the human α–subunit gene to overexpress hexosaminidase[27]. These murine NSC clones secrete functional hexosaminidase-A[27]. A transgenic mouse with an α-subunit deletion[27] permitted examination of the ability of human NSCs to secrete a gene product capable of rescuing TSD neural cells. NSCs (murine and human) were cocultured with dissociated TSD mouse brain cells from which they were separated by a porous membrane that allowed passage of hexosaminidase but not cells. After 10 days, the mutant neural cells were examined: (1) for the presence of hexosaminidase activity (Fig. 3A–C, and M); (2) with antibodies to the α-subunit and to CNS cell type markers to determine which TSD neural cells internalized corrective gene product (Fig. 3D–L); and (3) for reduction in GM_2 storage (Fig. 3N). While there was minimal intrinsic hexosaminidase activity in TSD cells cultured alone (Fig. 3A), hexosaminidase activity increased to normal intensity when the cells were cocultured with murine or human NSCs (Fig. 3B and C). The extent of human NSC-mediated cross-correction matched the success of murine NSCs, yielding percentages of hexosaminidase-positive TSD cells significantly greater than in untreated controls ($p<0.01$) (Fig. 3M). All neural cell types from the TSD mouse brain were corrected (Figs. 3D–L). The percentage of TSD CNS cells without abnormal GM_2 accumulation was significantly lower in those exposed to secretory products from human NSCs than in untreated TSD cultures ($p<0.01$), approaching those from wild-type mouse brain (Fig. 3N).

Multipotency and plasticity in vivo. We next determined whether human NSC clones (whether epigenetically or genetically propagated) could respond appropriately to normal developmental cues in vivo, which include migrating appropriately; integrating into host parenchyma; and differentiating into neural cell types appropriate to a given region's stage of development, even if that stage is not the one in which the NSCs were obtained. Although there are many approaches for testing these qualities[28–30], we used paradigms similar to those we have used with murine NSCs to assess their developmental ability[4]. When murine NSC clones are implanted into the cerebral ventricles of newborn mice, the cells engraft in the subventricular germinal zone (SVZ)[31] and follow the

established pathways used by endogenous progenitors, either migrating along the rostral migratory stream (RMS) to the olfactory bulb (OB), becoming neurons[12], or migrating into subcortical and cortical regions (where gliogenesis predominates and neurogenesis has ceased) becoming oligodendroglia and astroglia[11]. When transplanted into the germinal zone of the neonatal mouse cerebellum (the external germinal layer [EGL]), these same NSCs migrate inward and differentiate into granule neurons in the emerging internal granule cell layer (IGL)[10]. Following intraventricular implantation, human NSC clones emulated the developmentally appropriate behavior of their murine counterparts (Fig. 4 and 5). The engraftment, migration, and differentiation of epigenetically perpetuated clones were identical to that of *v-myc* perpetuated clones. Three of the five *v-myc* clones engrafted well (Table 1).

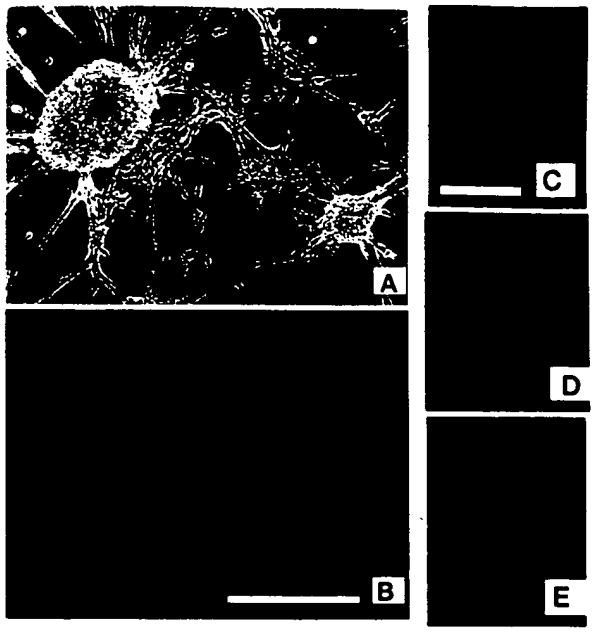

Figure 2. Characterization of human NSCs in vitro. (A) NSCs grown in serum-free medium. Immunostaining for (B) the neuronal marker neurofilament or (C) the oligodendroglia marker CNPase in serum-containing medium. (D) Immunostaining for the astrocyte marker human GFAP upon coculture with primary murine CNS cultures. (E) Immunostaining for the immature neural marker vimentin at transfer to serum-containing medium.

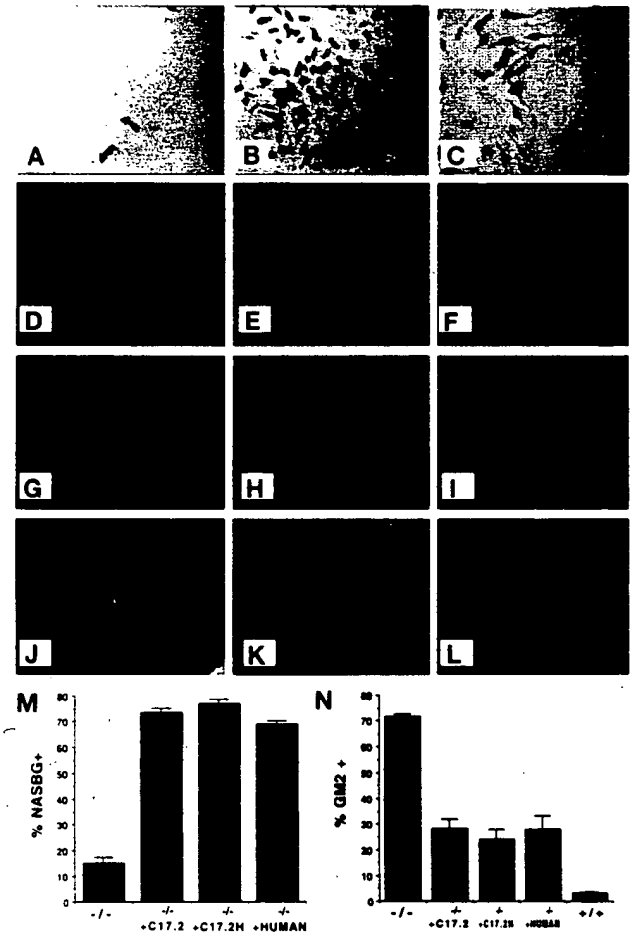

Figure 3. Dissociated brain cells from mice with mutated α-subunit of β-hexosaminidase (Tay-Sachs disease) cocultured with human NSCs. (A–C) Hexosaminidase activity determined by NASBG histochemistry. (A) TSD neural cells (arrows) not exposed to NSCs. TSD cells exposed to secretory products from (B) murine NSC clone C17.2H or from (C) human NSCs. (D–L) TSD cells cocultured with human NSCs immunostained with a (D–F) fluorescein-labeled antibody to the human α-subunit of β-hexosaminidase and (G–I) with antibodies to neural cell type-specific antigens. (G) Neuronal-specific NeuN marker; (H) glial specific GFAP marker; and (I) precursor maker, nestin. (J–L) Dual filter microscopy of the α-subunit and cell-type markers. (M) Percentage of β-hexosaminidase positive TSD cells; -/-: TSD α-subunit–null cells; TSD cells exposed to secretory products from C17.2+ murine NSCs; C17.2H+: murine NSC engineered to overexpress murine hexosaminidase; +human: human NSCs. (N) GM_2 accumulation in TSD cells; labels as in (M); +/+: wild-type mouse brain.

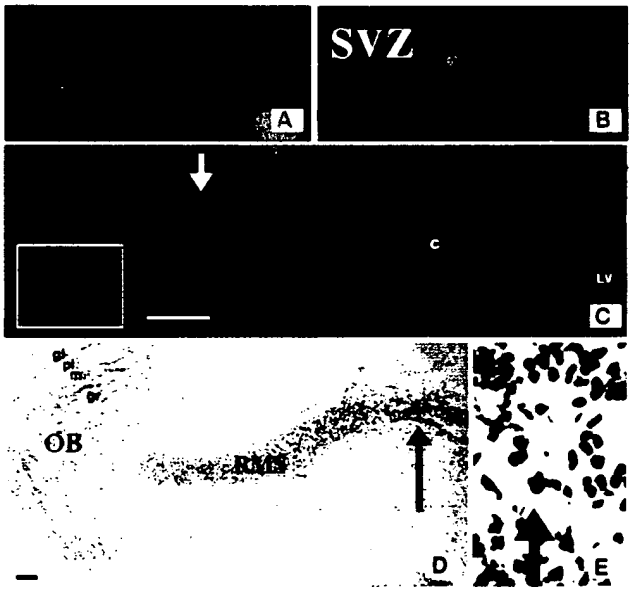

Figure 4. Migration of human NSCs following engraftment into the SVZ of newborn mice. (A,B) Human NSCs 24 h after transplantation. (A) Donor-derived cell (red) interspersed with (B) densely packed endogenous SVZ cells, visualized by DAPI (blue) in the merged image. (C) Donor-derived cells (red) within the subcortical white matter (arrow) and corpus callosum (c) and their site of implantation in the lateral ventricles (LV). Arrow indicates the cell shown at higher magnification within the inset. (D) Donor-derived cell migration from the SVZ into the rostral migratory stream (RMS) leading to the olfactory bulb (OB), in a cresyl-violet counterstained parasagittal section; gl: glomerular layer; pl: plexiform layer; m: mitral layer; gr: granular layer. Scale bars: 100 μm. (E) Higher magnification of area indicated by the arrow in (D). Brown staining indicates BrDU-immunoperoxidase-positive donor-derived cells.

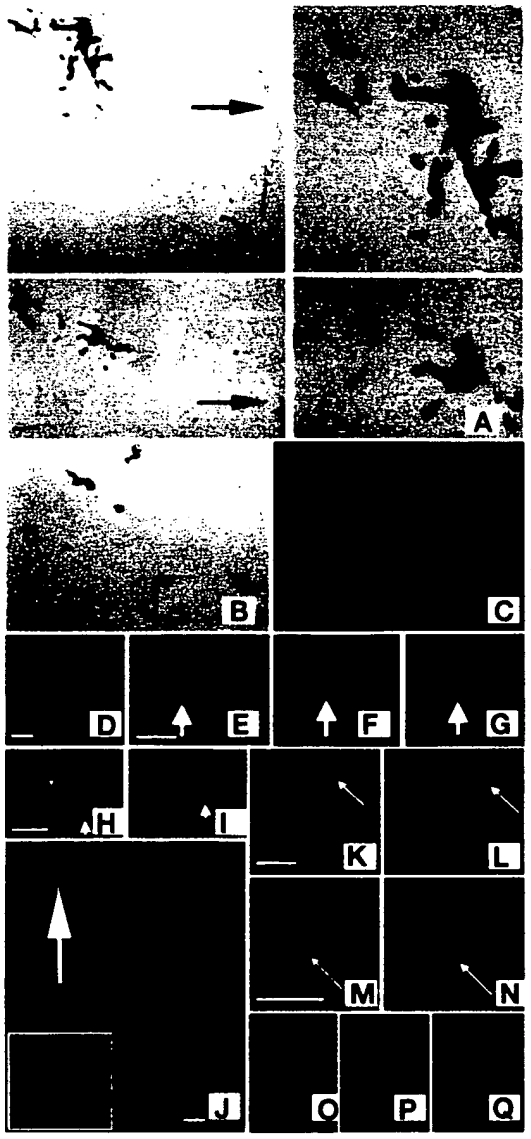

Figure 5. Characterization of human NSC clones in vivo following engraftment into SVZ of neonatal mice. (A–C) *LacZ*-expressing donor-derived cells from human NSC clone H1 detected with (A,B) Xgal and with (C) anti-β-galactosidase within (A) the periventricular and subcortical white matter regions and (B,C) OB granule layer. The arrows in (A) indicate the lateral ventricles. (D–G) BrdU-labeled NSCs (clone H6) implanted into the SVZ at birth identified in the OB with a (D) human-specific NF antibody and by (E–G) BrdU ICC via confocal microscopy. (E) BrdU-positive cell visualized by fluorescein; (F) anti-NeuN+ antibody visualized by Texas Red; (G) same cell visualized by dual filter. Donor-derived clone H6 in the adult subcortical white matter double-labeled with (H) an oligodendrocyte-specific antibody to CNPase and (I) BrdU. The arrowhead in (H) indicates a cytoplasmic process extending from the soma. (J) Donor-derived astrocytes (clone H6) in the adult subcortical white matter (indicated by the arrow) and striatum following neonatal intraventricular implantation, immunostained with a human astrocyte-specific anti-GFAP antibody. Inset is higher magnification. (K–Q) Expression of *v-myc* by human NSC clone H6 (K–N) 24 hours and (O–Q) 3 weeks following engraftment in the SVZ. (K,M,O) DAPI nuclear stains of the adjacent panels (L,N,P), immunostained for *v-myc* and (Q) immunostained for BrdU-positive donor-derived cells. (Q) is same as (P). Scale bars: (A and K): 100 μm; (D and E): 10 μm; (O): 50 μm.

Human NSCs integrated into the SVZ within 48 h following implantation (Figs. 4A and B, 5K–N). As with endogenous SVZ progenitors, engrafted human NSCs migrated out along the subcortical white matter by 2 weeks following engraftment (Fig. 4C), and, by 3–5 weeks had appropriately differentiated into oligodendrocytes and astrocytes (Fig. 5A and H–J). The ready detection of donor-derived astrocytes in vivo (Fig. 5J) contrasts with the initial absence of mature astrocytes when human NSC clones were maintained in vitro in isolation from the in vivo environment (Fig. 2D). Signals emanating from other components of the murine CNS appear necessary for promoting astrocyte differentiation and/or maturation from multipotent cells.

Endogenous SVZ progenitors also migrate anteriorly along the RMS and differentiate into OB interneurons[35]. By 1 week following transplantation, a subpopulation of donor-derived human cells from the SVZ migrated along the RMS (Fig. 4D and E). In some cases, these cells migrated together in small groups (Fig. 4E), a

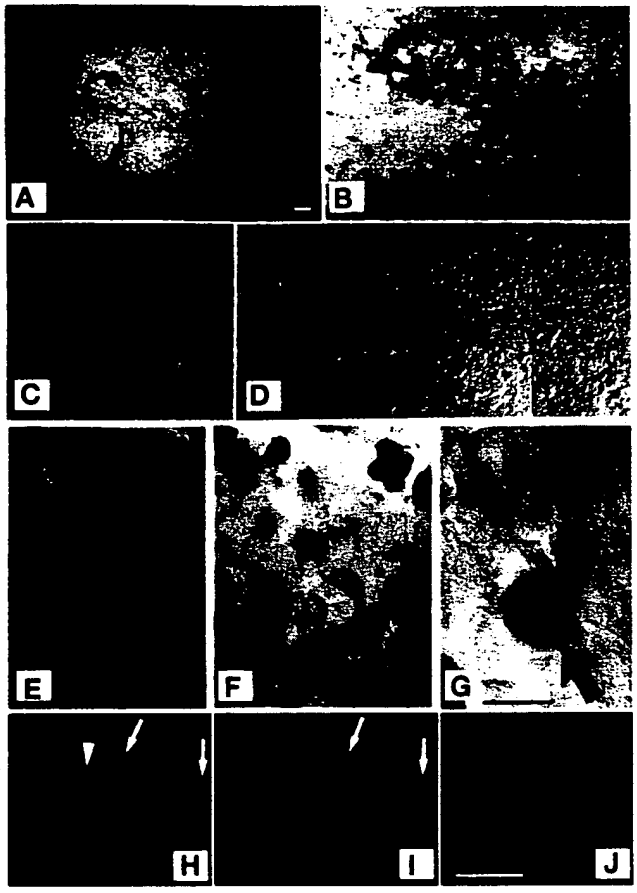

Figure 6. Transplantation of human NSCs into granule neuron-deficient cerebellum. (A–G) Donor-derived cells (clone H6) identified in the mature cerebellum by anti-BrDU immunoperoxidase cytochemistry (brown nuclei) following implantation into and migration from the neonatal *mea* EGL. (A) The internal granule cell layer (IGL and arrowheads) within the parasagittal section of the cerebellum. (B) Higher magnification of the posterior lobe indicated by "b" in (A). (C–G) Increasing magnifications of donor-derived cells within the IGL of a *mea* anterior lobe (different animal from [A,B]). (G) Normarski optics: residual host granule neurons indicated by arrowheads, representative BrDU positive donor-derived neuron indicated by the arrow. (H) Colabeling with anti-BrDU (green) and (I) NeuN (red) indicated with arrows. Arrowhead indicates BrDU+/NeuN- cell. (J) Fluorescent in situ hybridization of cells within the IGL using a human-specific probe (red). Scale bars: (A and B): 100 μm; (F, G, and J): 10 μm.

Table 1. Human neural stem cell clones.

Clone	Propagation technique	Engraftable
H1	bFGF	+
H6	v-*myc*	+
H9	v-*myc*	+
E11	v-*myc*	+
D10	v-*myc*	-
C2	v-*myc*	-

behavior typical of endogenous murine SVZ precursors[22]. Three weeks following transplantation, a subpopulation of donor-derived neurons (human-specific NF-positive cells) were present within the parenchyma of the OB, intermingled with host neurons (Fig. 5B–G). Not only were these donor-derived cells human NF-positive (Fig. 5D), but, when sections through the OB were reacted with both an antibody against BrDU (to identify prelabeled donor-derived human cells) and with an antibody to the mature neuronal marker NeuN, a large number of double-labeled BrDU+/NeuN+ donor-derived cells were integrated within the granule layer (Fig. 5E–G), mimicking the NeuN expression pattern of endogenous, host, murine interneurons (Fig. 5F and G).

Identical clones were implanted into a different germinal zone at the opposite end of the neuraxis to determine their plasiticity. Transplants of the same human NSCs into the EGLs of newborn mouse cerebella appropriately yielded different neuronal cell types in this different location, primarily cerebellar granule cells in the IGL (Fig. 6A–I), detailed below.

Therefore, in vivo—as in vitro (Fig. 2)—all engraftable human NSC clones gave rise to cells in all three fundamental neural lineages: neurons (Figs. 5D–G and 6], oligodendrocytes (Fig. 5H and I), and astrocytes (Fig. 5J). Not only did transplanted brains look histologically normal (donor cells migrated and integrated seamlessly into host parenchyma yielding no discernible graft margins), but engrafted animals exhibited no indications of neurologic dysfunction. Thus, structures that received contributions from donor human NSCs appeared to have developed normally.

Although most clones engrafted well, two appeared to engraft poorly (Table 1). Nevertheless, in vitro these clones displayed characteristics seemingly identical to those of the more robustly engrafting clones. Thus, ostensibly equivalent multipotency in vitro does not necessarily translate into equivalent potential in vivo, suggesting that each clone should be individually tested. This observation also suggests that transplantation of mixed polyclonal populations, because of their shifting representations of various clones, may be a problematic strategy.

Foreign transgene expression in vivo. Many CNS gene therapy needs require that donor cells express foreign genes in widely disseminated locations[28] (in addition to being able to do so in anatomically restricted regions[14]). Murine NSC clones have this capacity[21,22]. Human NSCs appear similarly capable. A representative retrovirally transduced, *lacZ*-expressing clone (Fig. 5A–C) continued to produce β–galactosidase after migration to, and stable integration and maturation within, host parenchyma at distant sites in the mature animal.

Spontaneous constitutive downregulation of *v-myc* expression. In the case of genetically manipulated human NSC clones, the propagating gene product *v-myc* is undetectable in donor human cells beyond 24–48 h following engraftment (Fig. 5K–Q) despite the fact that the brains of transplant recipients contain numerous stably engrafted, healthy, well-differentiated, nondisruptive, donor-derived cells (Figs. 4, 5A–J and Q, and 6). Identical findings have been observed with *v-myc*–propagated murine NSC clones[4] in which *v-myc* downregulation occurs constitutively and spontaneously and correlates with the typical quiescence of engrafted cells within 24–48 h posttransplantation. These observations suggest that *v-myc* is regulated by the normal developmental mechanisms that downregulate endogenous cellular *myc* in CNS precursors during mitotic arrest and/or differentiation. The loss of *v-myc* expression from stably engrafted NSCs following transplantation is consistent with the invariant absence of brain tumors derived from implanted *v-myc*–propagated NSCs, even after several years in mice[4,10]. As with mouse NSCs, neoplasms are never seen using human NSCs.

Neural cell replacement in vivo. Neurologic mouse mutants have provided ideal models for testing specific neural cell replacement strategies. The *meander tail* (*mea*) mutant is one such model of neurodegeneration and impaired development. *Mea* is characterized by a cell-autonomous failure of granule neurons to develop and/or survive in the cerebellum, especially in the anterior lobe[14]. Murine NSCs are capable of reconstituting the granule neuron-deficient IGL[14]. To assess whether human cells may be comparably effective in replacing neurons in CNS disorders, human NSC clones were engrafted into EGLs of newborn *mea* cerebella. When analyzed at the completion of cerebellar organogenesis, donor-derived human cells were present throughout the IGL (Fig. 6). They possessed the definitive size, morphology, and location of cerebellar granule neurons (Fig. 6E–G), identical to the few residual endogenous murine host granule neurons with which they were intermixed (Fig. 6G). That these replacement neurons were of human origin was confirmed by fluorescence in situ hybridization (FISH), using a human-specific chromosomal probe (Fig. 6J). The neuronal phenotype was confirmed by demonstrating that most engrafted cells in the *mea* IGL were immunoreactive for NeuN (Fig. 6H and I); as in the OB, endogenous interneurons in the IGL similarly express NeuN. Thus, engrafted NSCs of human origin appear sufficiently plastic to respond appropriately to varying local cues for lineage determination; recall that the donor human cells were not initially derived from a postnatal brain or from a cerebellum. Furthermore, human NSCs may be capable of appropriate neural cell replacement, much as murine NSCs are[34,35]. While many gene therapy vehicles depend on relaying new genetic information through established neural circuits—that may, in fact, have degenerated—NSCs may participate in the reconstitution of these pathways.

We have presented evidence that neural cells with stem cell features may be isolated from human brains and emulate NSCs in lower mammals[14], vouchsafing conservation of neurodevelopmental principles and suggesting that this cell type may be applied to a range of research and clinical problems in humans. NSCs may serve as adjuncts to other cellular[36], viral[37], and nonviral[37] vectors, including other human-derived neural cells[36–39]. Not only might the clones described here serve these functions, but our data suggest that investigators may readily utilize NSCs from other human material via a variety of equally safe and effective epigenetic and genetic means. That the methods used here yielded comparable cells suggests that investigators may choose the technique that best serves their needs. Insights from studies of NSCs perpetuated by one strategy may be legitimately joined to those derived from studies using others, providing a more complete picture of NSC biology and its applications.

Experimental protocol

Maintenance and propagation of human NSCs in culture. A suspension of primary dissociated neural cells (5×10^5 cells/ml), initially prepared and stably cultured from the periventricular region of the telencephalon of a 15-week human fetus[4] was plated on uncoated tissue culture dishes (Corning, Cambridge, MA) in the following growth medium: Dulbecco's Modified Eagles Medium (DMEM) + F12 medium (1:1) supplemented with N2 medium (Gibco, Grand Island, NY) to which was added bFGF (10–20 μg/ml) + heparin (8 μg/ml) and/or EGF (10–20 μg/ml). Medium was changed every 5

days. Cell aggregates were dissociated in trypsin-EDTA (0.05%) when >10 cell diameters in size and replated in growth medium at 5 x 10^5 cells/ml.

Differentiating culture conditions. Dissociated NSCs were plated on poly-L-lysine (PLL)-coated slides (Nunc, Naperville, IL) in DMEM + 10% fetal bovine serum (FBS) and processed weekly for immunocytochemistry (ICC). In most cases, differentiation occurred spontaneously. For astrocytic maturation, clones were cocultured with primary dissociated embryonic CD-1 mouse brain[20].

Retrovirus-mediated gene transfer. Two xenotropic, replication-incompetent retroviral vectors were used to infect human NSCs. A vector encoding *lacZ* was similar to BAG[10,20] except for the PG13 xenotropic envelope. An amphotropic vector encoding *v-myc* was generated using the ecotropic vector described for generating murine NSC clone C17.2 (ref. 20) to infect the GP + envAM12 amphotropic packaging line[21]. No helper virus was produced. Infection of bFGF- and/or EGF-maintained human neural cells with either vector (4×10^5 colony-forming units) was as described[10,20].

Cloning of human NSCs. Cells were dissociated, diluted to 1 cell/15 µl and plated at 15 µl/well of a Terasaki or 96-well dish. Wells with single cells were immediately identified. Single-cell clones were expanded and maintained in bFGF-containing growth medium. Monoclonality was confirmed by identifying a single and identical genomic insertion site by Southern blot analysis for either the *lacZ*- or the *v-myc*–encoding provirus in all progeny as described[20]. The *v-myc* probe was generated by nick translation labeling with ^{32}P dCTP; a probe to the *neo* sequence of the *lacZ*-encoding vector was generated by PCR using ^{32}P dCTP.

Cryopreservation. Trypsinized human cells were resuspended in a freezing solution comprising 10% dimethyl sulfoxide, 50% FBS, and 40% bFGF-containing growth medium and brought slowly to -140°C.

Cross-correction of mutation-induced β-hexosaminidase deficiency. The murine NSC clones C17.2 and C17.2H (ref. 22) were maintained in similar serum-free conditions as the human cells. NSCs were cocultured in a transwell system with primary dissociated neural cultures[19] from the brains of either wild-type or α-subunit null (TSD) neonatal mice[23]. These cultures were prepared under serum-free conditions, plated onto PLL-coated glass coverslips, and maintained in the medium described for NSCs. To assess production of a secretable gene product capable of rescuing the mutant phenotype, NSCs (murine and human) were cultured on one side of a membrane with 0.4 µm pores (sufficient to allow passage of hexosaminidase but not cells). The membrane was immersed in a well at the bottom of which rested the coverslip. After 10 days, coverslips were examined for hexosaminidase activity; for expression of the α-subunit in cells of various CNS lineages; for reduction in GM_2 storage. Hexosaminidase activity was assayed by standard histochemical techniques using the substrate napthol-AS-BI-*N*-acetyl-β-D-glucuroniside (NASBG)[24]; cells stain increasingly pink-red in direct proportion to their enzyme activity. NASBG staining of dissociated wild-type mouse brain cells served as a positive control for both intensity of normal staining and percentage of NASBG-positive cells (~100%). Neural cell types were identified by ICC with antibodies to standard markers: for neurons, NeuN (1:100; gift of R. Mullen, Chemicorp, Temecula, CA); for astrocytes, GFAP (1:500; Sigma, St. Louis, MO); for oligodendrocytes, CNPase (1:500; Sternberger Monoclonals, Baltimore, MD); and for immature undifferentiated progenitors, nestin (1:1000; Pharmingen, San Diego, CA). The α-subunit of human β-hexosaminidase was detected with a specific antibody[25]. Cells were assessed for dual immunoreactivity to that antibody and to the cell type-specific antibodies to assess which TSD CNS cell types had internalized enzyme from human NSCs. Intracytoplasmic GM_2 was recognized by a specific antibody[26].

Transplantation. For some models, each lateral ventricle of cryoanesthetized postnatal day 0 (P0) mice was injected as described[27] with 2 µl of NSCs suspended in phosphate buffered saline (PBS) (4×10^4 cells/µl). For other models, 2 µl of the NSC suspension were implanted into the EGL of each cerebellar hemisphere and the vermis as described[19]. All transplant recipients and untransplanted controls received daily cyclosporin 10 mg/kg given intraperitoneally (Sandoz, East Hanover, NJ) beginning on day of transplant. CD1 and *mea* mouse colonies are maintained in our lab.

Detection and characterization of donor human NSCs in vivo. Brains of transplanted mice were fixed and cryosectioned as described[10] at serial time points: P1, P2, and weekly through 5 weeks of age. Prior to transplantation, some human cells were transduced with *lacZ*. To control for and circumvent the risk of transgene downregulation, cells were also prelabeled either by in vitro exposure to BrDU (20 µM; 48 h prior to transplantation) and/or with the nondiffusible vital fluorescent membrane dye PKH-26 (immediately prior to transplantation as per Sigma protocol). Engrafted cells were then detected, as appropriate, by Xgal histochemistry[19]; by ICC with antibodies against β-galactosidase[28] (1:1000, XXX, Durham, NC), BrDU (1:10; Boehringer, Indianapolis, IN), human-specific NF (1:150; Boehringer), and/or human-specific GFAP (1:200; Sternberger Monoclonals); by FISH using a digoxigenin-labeled probe complementary to regions of the centromere present uniquely and specifically on all human chromosomes (Oncor, Gaithersburg, MD); and/or by PKH-26 fluorescence (through a Texas Red [TR] filter), with nondiffusibility having been verified for NSCs. Cell type identity of donor-derived cells was also established as necessary by dual staining with antibodies to neural cell type-specific markers: anti-NF (1:250; Sternberger) and anti-NeuN (1:20) to identify neurons; anti-CNPase (1:200–1:500) to identify oligodendrocytes; and anti-GFAP (1:150) to identify astrocytes. Immunostaining used standard procedures[29] and a TR-conjugated secondary antibody (1:200; Vector, Burlingame, CA). Immunoreactivity to human-specific antibodies also used standard procedures and a fluorescein-conjugated antimouse IgG secondary antibody (1:200; Vector). To reveal BrDU-intercalated cells, tissue sections were first incubated in 2N HCl (37°C for 30 min), washed twice in 0.1 M sodium borate buffer (pH 8.3), washed thrice in PBS, and permeabilized before exposure to anti-BrDU. Immunoreactivity was revealed with either a fluorescein-conjugated (1:250; Jackson, West Grove, PA) or a biotinylated (1:200; Vector) secondary antibody. *V-myc* expression (unique to donor-derived cells) was assessed with an antibody to the protein (1:1000; UBI, Lake Placid, NY). To visualize cellular nuclei, sections were incubated in the blue fluorescent nuclear label DAPI (10 min at 20°C). FISH for the human-specific centromere probe was performed on cryosections from 4% paraformaldehyde/2% glutaraldehyde-fixed brains that were permeabilized, incubated in 0.2 N HCl, exposed to proteinase K (100 µg/ml in 0.1M Tris, 0.005 M EDTA [pH 8.0]), washed (0.1% glycine), and rinsed (50% formamide/2× SSC). Probe was then added to the sections, which were coverslipped, denatured (100°C for 10 min), hybridized (15 h at 37°C), and washed (per manufacturer's protocol). Probe was detected by an antidigoxigenin TR-conjugated antibody (Boehringer) diluted 1:5 in 0.5% bovine serum albumin + 5% normal human serum in PBS. For some donor cells, multiple detection techniques were performed.

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1. McKay, R.D.G. 1997. Stem cells in the central nervous system. *Science* **276**:66–71.
2. Gage, F.H. and Christen, Y. (eds.). 1997. *Isolation, characterization, and utilization of CNS stem cells—research & perspectives in neuroscience.* Springer-Verlag, Heidelberg, Berlin.
3. Morrison, S.J., Shah, N.M., and Anderson, D.J. 1997. Regulatory mechanisms in stem cell biology. *Cell* **88**:287–298.
4. Stemple, D.L. and Mahanthappa, N.K. 1997. Neural stem cells are blasting off. *Neuron* **18**:1–4.
5. Weiss, S., Reynolds, B.A., Vescovi, A.L., Morshead, C., Craig, C., and van der Kooy, D. 1996. Is there a neural stem cell in the mammalian forebrain. *Trends Neurosci.* **19**:387–393.
6. Alvarez-Buylla, A. and Lois, C. 1995. Neuronal stem cells in the brain of adult vertebrates. *Stem Cells (Dayton OH)* **13**:263–272.
7. Qian, X., Davis, A.A., Goderie, S.K., and Temple, S. 1997. FGF2 concentration regulated the generation of neurons and glia from multipotent cortical stem cells. *Neuron* **18**:81–93.
8. Snyder, E.Y. 1998. Neural stem-like cells: Developmental lessons with therapeutic potential. *The Neuroscientist.* In press.
9. Martinez-Serrano, A. and Bjorklund, A. 1997. Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci.* **20**:530–538.
10. Snyder, E.Y., Deitcher, D.L., Walsh, C., Arnold-Aldea, S., Hartweig, E.A., and Cepko, C.L. 1992. Multipotent cell lines can engraft and participate in the development of mouse cerebellum. *Cell* **68**:1–20.
11. Renfranz, P.J., Cunningham, M.G., and McKay, R.D.G. 1991. Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* **66**:713–729.
12. Shihabuddin, L.S., Hertz, J.A., Holets, V.R., and Whittemore, S.R. 1995. The adult CNS retains the potential to direct region-specific differentiation of a transplanted neuronal precursor cell line. *J. Neurosci.* **15**:6666–6678.
13. Gage, F.H., Coates, P.W., and Palmer, T.D. 1995. Survival and differentiation of

adult neuronal progenitor cells transplanted to the adult brain. *Proc. Natl. Acad. Sci. USA* **92**:11879–11883.
14. Fisher, L.J. 1997. Neural precursor cells: application for the study and repair of the central nervous system. *Neurobiol. Dis.* **4**:1–22.
15. Whittemore, S.R. and Snyder, E.Y. 1996. The physiologic relevance and functional potential of central nervous system-derived cell lines. *Mol. Neurobiol.* **12**:13–38.
16. Gage, F.H. 1998. Cell therapy. *Nature* (suppl.) **392**:18–24.
17. Verma, I.M. and Somia, N. 1997. Gene therapy: promises, problems, and prospect. *Nature* **389**:239–242.
18. Kilpatrick, T. and Bartlett, P.F. 1993. Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. *Neuron* **10**:255–265.
19. Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E. et al. 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci.* **16**:1091–1100.
20 Ryder, E.F., Snyder, E.Y., and Cepko, C.L. 1990. Establishment and characterization of multipotent neural cell lines using retrovirus vector mediated oncogene transfer. *J. Neurobiol.* **21**:356–375.
21. Snyder, E.Y., Taylor, R.M., and Wolfe, J.H. 1995. Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain. *Nature* **374**:367–370.
22. Lacorazza, H.D., Flax, J.D., Snyder, E.Y., and Jendoubi, M. 1996. Expression of human β-hexosaminidase α-subunit gene (the gene defect of Tay-Sachs disease) in mouse brains upon engraftment of transduced progenitor cells. *Nat. Med.* 4:424–429.
23. Kitchens, D.L., Snyder, E.Y., and Gottlieb, D.I. 1994. bFGF and EGF are mitogens for immortalized neural progenitors. *J. Neurobiol.* **25**:797–807.
24. Kornblum, H.I., Raymon, H.K., Morrison, R.S., Cavanaugh, K.P., Bradshaw, R.A., and Leslie, F.M. 1990. Epidermal growth factor and basic fibroblast growth factor: effects on an overlapping population of neocortical neurons in vitro. *Brain Res.* **535**:255–263.
25. Birren, S.J., Verdi, J.M., and Anderson, D.J. 1992. Membrane depolarization induces p140trk and NGF responsiveness, but not p75LNGFR, in MAH cells. *Science* **257**:395–397.
26. Snyder, E.Y. and Wolfe, J.H. 1996. CNS cell transplantation: a novel therapy for storage diseases? *Current Opin. Neurol.* **9**:126–136.
27. Yamanaka, S., Johnson, M.D., Grinberg, A., Westphal, H., Crawley, J.N., Taniike, M. et al. 1994. Targeted disruption of the HexA gene results in mice with biochemical and pathologic features of Tay-Sachs disease. *Proc. Natl. Acad. Sci. USA* **91**:9975–9979.
28. Suhonen, J.O., Peterson, D.A., Ray, J., and Gage, F.H. 1996. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature* **383**:624–627.
29. Fishell, G. 1995. Striatal precursors adopt cortical identities in response to local cues. *Development* **121**:803–812.
30. Campbell, K., Olsson, M., and Bjorklund, A. 1995. Regional incorporation and site-specific differentiation of striatal precursors transplanted to the embryonic forebrain ventricle. *Neuron* **15**:1259–1273.
31. Sidman, R.L., Miale, I.L., and Feder, N. 1959. Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. *Exp. Neurol.* **1**:322–333.
32. Lois, C., Garcia-Verdugo, J.-M., and Alvarez-Buylla, A. 1996. Chain migration of neuronal precursors. *Science* **271**:978–981.
33. Goldman, S.A. and Luskin, M.B. 1998. Strategies utilized by migrating neurons of the postnatal vertebrate forebrain. *Trends Neurosci.* **21**:107–114.
34. Rosario, C.M., Yandava, B.D., Kosaras, B., Zurakowski, D., Sidman, R.L., and Snyder, E.Y. 1997. Differentiation of engrafted multipotent neural progenitors towards replacement of missing granule neurons in meander tail cerebellum may help determine the locus of mutant gene action. *Development* **124**:4213–4224.
35. Snyder, E.Y., Yoon, C.H., Flax, J.D., and Macklis, J.D. 1997. Multipotent neural progenitors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. *Proc. Natl. Acad. Sci. USA* **94**:11645–11650.
36. Svendsen, C.N., Caldwell, M.A., Shen, J., ter Borg, M.G., Rosser, A.E., Tyers, P. et al. 1997. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Exp. Neurol.* **148**:135–146.
37. Sabaate, O., Horellou, P., Vigne, E., Colin, P., Perricaudaet, M., Buc-Caron, M.-H. et al. 1995. Transplantation to the rat brain of human neural progenitors that were genetically modified using adenovirus. *Nat. Genet.* **9**:256–260.
38. Borlongan, C.V., Tajima, Y., Trojanowski, J.Q., Lee, V.M., and Sanberg, P.R. 1998. Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Exp. Neurol.* **149**:310–321.
39. Sah, D.W.Y., Ray, J., and Gage, F.H. 1997. Bipotent progenitor cell lines from the human CNS. *Nat. Biotechnol.* **15**:574–580.
40. Moretto, G., Xu, R.Y., Walker, D.G., and Kim, S.U. 1994. Co-expression of mRNA for neurotrophic factors in human neurons and glial cells in culture. *J Neuropathol. Exp. Neurol.* **53**:78–85.
41. Markowitz, D., Goff, S., and Bank, A. 1988. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* **167**:400–406.

EXHIBIT B

STEM CELL BIOLOGY

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9

Primordial Germ Cells as Stem Cells

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Germ cells are the precursors of the mature gametes, making their status as stem cells apparently unassailable. The fusion of the gametes to produce a totipotent zygote initiates the whole program of embryonic development, leading to the formation of the stem cells of all adult tissues as well as the next generation of germ cells. Focusing on mammals, in this review, I examine how germ cells arise and whether their precursors are stem cells in their own right. I also discuss how the study of germ cells and their precursors sheds light on the important questions of what controls pluripotency and how genomes are reprogrammed. For the purposes of this review, I define stem cells as a cell population that has the capacity both to self-renew and to give rise to at least one kind of nondividing, fully differentiated descendant.

The germ cell lineage usually originates as a very small founding population that is segregated from somatic cells early in development, at least in organisms where the overall body plan is also established early (Dixon 1994). Perhaps the physical separation of germ cells from organizing centers helps to protect them from the influence of potent signaling factors and morphogenetic movements. In vertebrates and *Drosophila,* there is considerable proliferation of the founding population as it moves from its site of origin to the gonads. The term primordial germ cells (PGCs) is strictly applied to the diploid germ cell precursors that transiently exist in the embryo before they enter into close association with the somatic cells of the gonad and become irreversibly committed as germ cells. Male and female PGCs are indistinguishable, and in mammals both will finally stop dividing and enter into meiosis when associated with the somatic cells of the ovary, or even with tissues such as the adrenal gland outside the gonads. However, in the testis, PGCs behave differently, since they come under the influence of the XY gonadal cells

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that produce a short-range, diffusible, meiosis-inhibiting factor. Male PGCs therefore normally undergo mitotic arrest in G_1 as prospermatogonial stem cells that do not divide again until puberty (for review, see McLaren 1994; Sassone-Corsi 1997). Some limited proliferation of spermatogonial stem cells can be obtained in culture (Nagano et al. 1998). Therefore, the mammalian germ line consists of two distinct stem cell populations, the transient population of PGCs outside the gonad and the spermatogonial stem cells within, that self-renew and differentiate into sperm throughout the fertile life of the adult male (Fig. 1).

THE ORIGIN OF PRIMORDIAL GERM CELLS: INHERITANCE OF CYTOPLASMIC DETERMINANTS VERSUS INDUCTION BY EXTRINSIC FACTORS

In most organisms studied, but with several exceptions including mammals and birds (Dixon 1994), the segregation of pluripotent germ cells from somatic cells involves maternal factors or determinants. These are deposited in the cytoplasm of the egg and during cleavage are asymmetrically segregated into a small number of blastomeres that subsequently differentiate into PGCs. Germ cell determinants are complexes of RNA

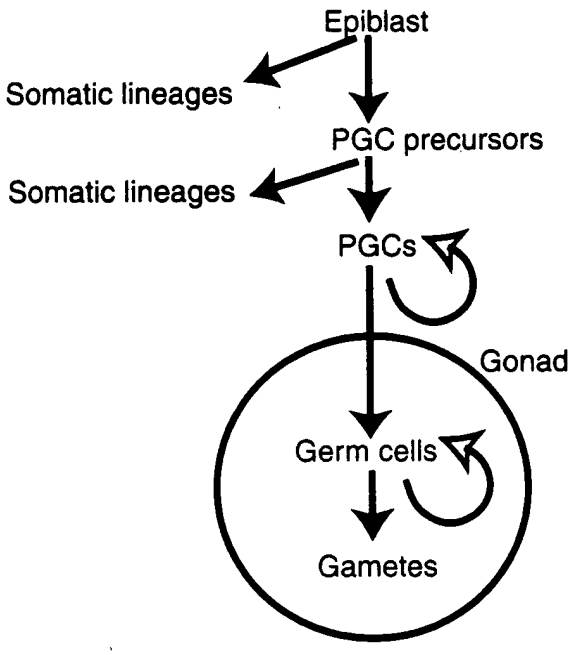

Figure 1 Germ cell lineage in mammals. Pluripotent cells (that express *Oct4*) are shown. Stem cell self-renewal is shown as a curved arrow. In the gonad only the male germ cells constitute a stem cell population; oocytes do not proliferate.

and protein that have been best characterized in *Caenorhabditis elegans* (where they associate into organelles called P granules) and in *Drosophila* (where they constitute the polar granules or pole plasm). For example, in *Drosophila*, components include oskar, nanos, vasa, and tudor. Ectopic expression of oskar in the *Drosophila* blastula is sufficient to initiate the formation of ectopic germ cells. However, in *C. elegans*, P granule components are necessary but not sufficient for the specification of germ cells (for review, see Ephrussi and Lehmann 1992; Hubbard and Greenstein 2000). Very little is known about the way in which germ plasm components regulate gene expression and cell behavior in germ cell precursors (for review, see Wylie 1999). In *C.elegans*, zygotic gene expression and possibly mRNA stability are repressed in germ-line blastomeres by at least one P granule component (the protein PIE-1) (for review, see Seydoux and Strome 1999). Other gene products, for example, the polycomb group MES proteins, are involved in transcriptional silencing at the level of chromatin structure. One hypothesis, therefore, is that a number of independent repression mechanisms protect germ cells in *C. elegans* from responding to signals that normally restrict the developmental options of somatic cell lineages. By shutting down gene expression, the germ cell lineage is kept pluripotential (for discussion, see Dixon 1994).

The properties and behavior of germ plasm in *Xenopus*, and similarities with *Drosophila* polar granules, have been thoroughly discussed previously (Wylie 1999). Homologs of vasa, a component of *Drosophila* polar granules, have recently been identified in zebrafish primordial germ cells (Braat et al. 1999; Weidinger et al. 1999).

Mammals and chick (Ginsberg 1994) apparently do not have maternally derived germ cell determinants. Mouse genes that encode homologs of *Drosophila* polar granule components, for example, vasa (*Mvh*) and germ cell-less (*Gcl*), are not expressed in PGCs but in adult male germ cells (Fujiwara et al. 1994; Leatherman et al. 2000). If maternally encoded germ plasm is absent from mammals, what regulates PGC formation? The current idea is that PGC precursors are induced in the embryo by secreted signaling factors produced by adjacent extraembryonic cells. It is still possible that the localized production of these inducing factors is under the control of maternal determinants segregated to extraembryonic cells, but this hypothesis has not yet been tested.

To enable critical evaluation of the induction of PGCs, a brief description of early mouse development is in order (Fig. 2). At around the time of implantation (~4.0–4.5 days post coitum, dpc) the blastocyst consists of two populations of cells; an outer epithelial layer of trophoblast

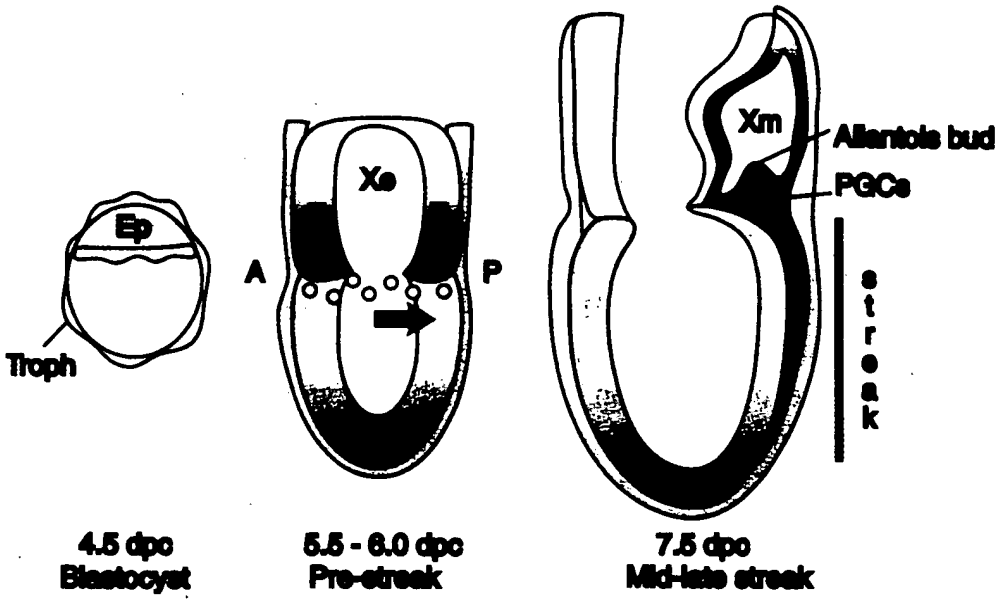

Figure 2 Model for the development of PGCs in the mouse embryo. At 4.5 dpc the blastocyst consists of an outer layer of trophoblast (Troph) surrounding the epiblast (Ep; *green*) and the primitive or visceral endoderm (*orange*). By 5.5–6.0 dpc, the epiblast has given rise to the embryonic ectoderm (Ect) and the trophoblast has formed the extraembryonic ectoderm (Xe). Signals from the Xe, for example BMP4 and probably BMP8b (*blue arrows*), are thought to induce PGC precursors (*open circles*) in the proximal epiblast. These move from anterior to posterior (A–P; *green arrow*). By 7.5 dpc, mesoderm has been generated (*red*) in the primitive streak and extraembryonic region (Xm). Descendants of the PGC precursors are allocated to either the extraembryonic mesoderm or PGC (*filled circles*) lineages.

cells that surrounds a tightly packed cluster of undifferentiated inner cell mass (ICM) cells. The ICM subsequently differentiates into an inner epiblast or embryonic ectoderm population and an outer primitive or extraembryonic visceral endoderm. After implantation, all cell types proliferate rapidly and the trophoblast forms a knob-like mass of cells known as the extraembryonic ectoderm, and the epiblast cells become organized into a cup-shaped epithelium. There is a clear morphological demarcation or junction between the epiblast and the extraembryonic ectoderm, and lineage analysis strongly suggests there is no mixing of cells between the two tissues after about 4.0 dpc. Around 6.0 dpc, the embryo begins to undergo gastrulation. Proximal epiblast cells move posteriorly, lose their epithelial organization, and give rise to unpolarized mesodermal cells. The first cells to delaminate from the epiblast give rise to extraembryonic mesoderm, whereas cells that drop out later give rise to embryonic

mesoderm in the primitive streak. As described in detail below, the first time PGCs can be clearly distinguished from somatic cells in the mouse embryo is around 7.5 dpc. A cluster of about 45–50 cells that express high levels of the genes encoding tissue nonspecific alkaline phosphatase (TNAP) and the OCT4 transcription factor can be identified posterior to the primitive streak at the base of the allantois. They are surrounded by somatic mesoderm cells that express much lower levels of these markers (see Anderson et al. 2000).

Several lines of evidence show that PGCs arise from cells in the epiblast, although the precise time at which the PGC progenitors are committed to their fate has not been determined. The first evidence comes from the elegant lineage analysis experiments of Kirstie Lawson (Lawson and Hage 1994). She injected single cells in the epiblast at the prestreak (pregastrulation) and early streak stages with a fluorescent lineage marker and then cultured the embryos for 40 hours and determined the location of labeled descendants. Analysis showed that PGCs (as judged by alkaline phosphatase staining) were derived from cells originally located in the proximal region of the prestreak epiblast, dispersed within about three cell diameters of the junction with the extraembryonic ectoderm. A crucial finding was that no injected epiblast cells gave rise to PGCs alone. Cells that generated labeled PGCs also gave rise to labeled cells in the extraembryonic mesoderm (most frequently allantois, but also amnion and extraembryonic mesoderm of the yolk sac). This finding showed that the so-called PGC precursor cell population must generate descendants committed to either the extraembryonic mesoderm or the germ cell lineages. It is thought that this allocation takes place when the precursors are posterior to the primitive streak at around 7.5 dpc, but nothing is known about the mechanisms involved. For example, the process may be stochastic and cell-autonomous or influenced by extrinsic factors. It may involve lateral inhibition, or asymmetric cell division and the localization of zygotic (rather than maternal) gene products to the PGC lineage. One limitation of the cell lineage analysis described above is that it does not say anything about the time at which the PGC precursors are first set aside. It only tells us that they already exist at 6.0 dpc in the prestreak epiblast. The process leading to the generation of the precursors could have been initiated significantly earlier.

The fact that PGC precursors are located in the epiblast close to the junction with the extraembryonic ectoderm suggests that this environment contains factors inducing PGC precursor fate. To test this hypothesis, Tam and Zhou (1996) carried out an embryonic grafting experiment. They isolated clumps of 5–20 distal epiblast cells from early streak stage embryos

of a reporter transgenic line that expresses β-galactosidase constitutively in all cells. The pieces were then grafted close to the junction with the extraembryonic ectoderm of wild-type, early streak stage (6.5 dpc) embryos, and the chimeric embryos were cultured in vitro. Analysis of these embryos showed many lacZ-positive cells in the extraembryonic and posterior mesoderm. In a very small number of embryos a few PGCs could be found that coexpressed alkaline phosphatase and β-galactosidase. This important experiment indicated that distal embryonic ectoderm cells, which would normally have developed into anterior ectoderm or neurectoderm, can change in response to exogenous signals and acquire more ventral posterior cell fates, including that of PGCs. However, it does not reveal the whole window of time when induction of PGC precursors normally takes place; it only tells us that the inducing activity is still available at the early streak stage. In fact, it may be that induction occurs over an extended period of time, with some epiblast cells receiving sufficient inducing signal early, even at the blastocyst stage, and others not until 6.5 dpc.

If mouse or human blastocysts are grown in vitro, the ICM can give rise to pluripotential embryonic stem (ES) cell lines that can both self-renew indefinitely and give rise to multiple cell types in culture. When mouse ES cells are injected into a blastocyst, they mix with the ICM cells and contribute to all the tissues of the embryo except for the trophoblast and extraembryonic ectoderm or extraembryonic visceral endoderm. Some of the ES cells are able to differentiate along the germ cell lineage. Since the ES cells have undergone extensive proliferation in culture, this finding argues strongly against maternally inherited factors in the epiblast or ICM playing a role in germ cell determination in mammals. However, the results again shed no light on when or how the induction of the germ cell precursors first takes place.

EVIDENCE THAT FACTORS PRODUCED BY THE TROPHOBLAST OR EXTRAEMBRYONIC ECTODERM PLAY A ROLE IN MAMMALIAN GERM-LINE DEVELOPMENT

The experiments described above suggest that germ-line-inducing factors are present near the junction between the trophoblast and ICM or extraembryonic ectoderm and epiblast. The first evidence for the nature of the factors came from the observation that embryos homozygous null for the gene encoding the transforming growth factor β (TGFβ)-related growth factor, bone morphogenetic protein 4 (BMP4), completely lack both an allantois (assessed morphologically) and PGCs (assessed by staining for either alkaline phosphatase or the carbohydrate antigen,

SSEA-1 [Lawson et al. 1999; N.R. Dunn and B.L.M. Hogan, unpubl.]). *Bmp4* is first expressed at high levels in the extraembryonic ectoderm and only later in the extraembryonic mesoderm cells in the allantois and posterior primitive streak surrounding the PGCs (Lawson et al. 1999). *Bmp4* is not expressed in the founding population of PGCs. The early expression pattern of *Bmp4* suggests that the protein secreted by the extraembryonic ectoderm induces cells in the proximal epiblast to assume the fate of PGC/allantois precursors. The finding that wild-type ES cells cannot rescue the mutant phenotype, even when they contribute more than 90% of the cells in chimeric embryos, supports this hypothesis. Finally, *Bmp4* heterozygous embryos have a significantly smaller founding population of PGCs than normal, even though, once formed, the cells proliferate at the same rate as wild-type PGCs. This is consistent with a model in which BMP4 produced by the extraembryonic ectoderm acts in a dose-dependent manner to control the fate of the pluripotent proximal epiblast cells. According to this model, epiblast cells that receive the highest dose of BMP4 over the longest period have a high probability of becoming PGC precursors, whereas cells receiving a lower dose are more likely to give rise to extraembryonic or lateral mesoderm.

Further experiments are needed to distinguish between this model and alternatives. For example, rather than acting instructively, BMP4 may function simply as a permissive factor, maintaining the survival of PGC precursors segregated by a different mechanism. One prediction of the instructive or morphogen model is that in *Bmp4* homozygous mutants (and in mutants of genes encoding receptors or components of downstream signaling pathways), the fate of cells in the proximal epiblast, including those that normally give rise to PGC precursors, is changed to more dorsal/anterior cell types. Another prediction is that BMP protein should induce PGC precursors in isolated epiblasts. However, it is possible that BMP4 is necessary but not sufficient for inducing PGC precursors, and that additional factors, including ones made by the visceral endoderm, are required. Recent data suggest that at least one factor secreted by the extraembryonic ectoderm functions in collaboration with BMP4 to control PGC development. This is the related protein, BMP8b, made exclusively by the extraembryonic ectoderm at this stage of development (Ying et al. 2000). BMP8b may act independently as a homodimer or possibly form biologically active heterodimers with BMP4, although the data do not support the hypothesis that such heterodimers are obligatory for PGC precursor formation. Finally, it is not yet known whether BMP4 and BMP8b act directly on the epiblast, or indirectly through the extraembryonic endoderm.

Further analysis of the role of cell–cell interactions in early PGC development would be greatly facilitated by the development of specific molecular markers both for PGC precursors before they have moved into the posterior primitive streak and for the descendants of these precursors that have differentiated along the PGC lineage.

CHARACTERISTICS OF MAMMALIAN PGCs BEFORE THEY REACH THE GONAD AND MAINTENANCE OF THE PLURIPOTENT STATE

The founding population of PGCs of the 7.5-dpc mouse embryo undergoes two important processes en route to their final resting place in the gonads. The cells proliferate, and they migrate along the endoderm of the hind gut, through the mesentery and into the genital ridges. Most PGCs have reached the ridges at 11.5 dpc, and proliferation ceases by about 13.5 dpc. Migration is common to germ cells of several organisms; e.g., *Drosophila*, *Xenopus*, zebrafish, and chick. However, since it is not obviously relevant to the stem cell status of PGCs, it will not be considered further here, and readers are referred to other reviews and papers (Wylie 1999; Anderson et al. 2000; Bendel-Stenzel et al. 2000). It should be noted, however, that chick PGCs migrate to the gonads via the bloodstream (Ginsberg 1994), but no intravascular PGCs have been seen in mammals, even though their migration route carries them near major blood vessels.

Proliferation increases the number of PGCs in the embryo from around 150 at 8.5 dpc to about 25,000 by 13.5 dpc (Tam and Snow 1981), giving a population doubling time of about 16 hours. It is not known whether the number of cell divisions undergone by each PGC in vivo is invariant.

Unlike hematopoietic stem cells, there are still very few molecular markers characteristic of mammalian PGCs. Indeed, no gene is yet known that is exclusively expressed in PGCs and PGC precursors. The markers that are most frequently used to distinguish PGCs after about 7.5–8.5 dpc are TNAP (MacGregor et al. 1995), stage-specific embryonic antigen-1 (SSEA1, a complex surface carbohydrate), and OCT4 (a POU-domain transcription factor).

OCT4 (encoded by the *Pou5f1* gene in mice and also known as OCT3/4) is of particular interest because it appears to be a key regulator of the pluripotential phenotype. It is expressed in all the cells of the cleavage-stage embryo and late-stage morula, but switched off in the trophoblast, and remains active in the embryonic ectoderm and primitive endoderm until gastrulation. It is then gradually down-regulated in the derivatives of the embryonic ectoderm and endoderm, and by 8.5 dpc is

only expressed in the PGCs. It is finally extinguished in the germ line when the PGCs begin to differentiate in the gonad, only to be reactivated as the gametes reach maturity (Pesce et al. 1998). This pattern of expression led to the hypothesis that OCT4 is a guardian of the pluripotential phenotype and prevents cells from becoming restricted in their developmental potential. This idea is supported by the observation that *Pou5f1* null embryos lack an inner cell mass and consist entirely of trophoblast cells (Nichols et al. 1998). More recent studies have suggested that the precise level of *Pou5f1* expression in undifferentiated ES cells regulates their differentiation in vitro (Niwa et al. 2000). Intermediate levels of OCT4 protein appear to favor the pluripotential, undifferentiated phenotype, whereas low levels promote the differentiation of ES cells into trophoblast, and high levels into endoderm and mesoderm. It is thought that OCT4 maintains the undifferentiated state by regulating gene transcription in collaboration with coactivators such as SOX2 or ROX-1. High levels of these cofactors may be maintained by signaling through the IL-6/LIF (leukemia inhibitory factor) receptor subunit, gp130, mediated by STAT-3 activation. As described in the next section, LIF is a cytokine that was recognized for its ability to maintain the undifferentiated state of ES cells in vitro. However, homozygous null *Lif* mutant embryos develop normally, so that if gp130 signaling plays a role in vivo, it must be activated by LIF-related cytokines. Obviously, an important goal is to identify genes up- or down-regulated by OCT4 in pluripotential epiblast cells and PGCs in vivo. One candidate is *Fgf4* (Ambrosetti et al. 1997); another is *Kit* (see below). It is noteworthy that OCT4 appears to regulate gene expression in mammalian PGCs rather differently from pole plasm determinants in *C.elegans*, which apparently function by generally repressing gene expression in the PGCs.

Mammalian PGCs are distinguished from somatic cells by a number of genome-wide modifications. Normally, during preimplantation development, zygotic DNA is demethylated, except at sites associated with allele-specific parental imprinting (Monk et al. 1987). Remethylation occurs in somatic cells before gastrulation, but the PGCs (and presumably their precursors) do not undergo this epigenetic modification. In addition, PGCs go one step further and remove the methylation of parentally imprinted loci that persists in somatic cells (Kato et al. 1999). This reprogramming appears to occur gradually, as the PGCs migrate to the genital ridges, and is completed by 13.5 dpc. New imprints are subsequently added during germ cell maturation. Erasure of parental imprinting in PGCs has two consequences. First, PGCs that have not yet begun their differentiation into mature germ cells are unique in having no modifica-

tion of their genome at all, at least at the level of DNA methylation (Kato et al. 1999). This may be necessary to erase the epigenetic influences or modifications of the parents and to restore the totipotency of the germ line. Second, PGCs late in the migratory pathway or just arrived in the gonad have a different phenotype from PGCs at 13.5 dpc. It could therefore be argued that the proliferation phase of PGCs does not strictly speaking involve a self-renewal, but rather the rapid amplification of a transitional precursor population.

Another difference in genomic modification between PGCs and somatic cells is the fact that female PGCs avoid random inactivation of their X chromosomes, at least during the early stages of their existence (Tam et al. 1994). Both X chromosomes are active in the epiblast until shortly before gastrulation, when a wave of random X inactivation goes through the population (Tan et al. 1993). However, at the earliest time they can be recognized, which is posterior to the primitive streak and in the hindgut endoderm, most PGCs still have two active X chromosomes. By the time they have reached the gonad, however, most have asynchronously undergone X inactivation. The X chromosomes are then reactivated before the onset of meiosis. Understanding how the PGCs initially avoid X inactivation will provide important information about the mechanism of germ-line specification at the genomic level.

PROLIFERATION OF PGCs

At least three different extracellular ligand/receptor signaling systems have been identified that promote the survival and proliferation of PGCs. These are (1) stem cell factor and its tyrosine kinase receptor, (2) bFGF and FGF receptors, and (3) cytokines of the interleukin/LIF family and their receptors that signal through a common gp130 subunit.

Stem cell factor (SCF, also known as Steel factor and mast cell growth factor) encoded by the *Mgf* (formerly *Steel*) locus, and its transmembrane tyrosine kinase receptor, c-KIT, encoded by the *Kit* (formerly *W*) gene, were first identified as growth factors for PGCs from genetic analysis in the mouse. *Mgf* is expressed in the somatic cells through which the PGCs migrate, whereas *Kit* is expressed by the PGCs themselves, at least until a few days after arrival in the genital ridge when it is down-regulated (Bendel-Stenzel et al. 2000; Manova and Bachvarova 1991).

A role for FGFs and receptors (e.g., FGFR1 and 2) in promoting PGC proliferation was first suggested from experiments in which purified bFGF was added to cells in culture (Matsui et al. 1992; Resnick et al. 1998). Whether FGFs play a role in vivo is not known, but *Fgf 3,4, 5,* and

8 genes are variously expressed in the epiblast, posterior primitive streak, and mesoderm along the migration route of the PGCs.

Other factors that promote the survival and proliferation of PGCs in vitro are leukemia inhibitory factor (LIF), oncostatin M (OSM), interleukin-6 (IL-6), and ciliary neurotrophic factor (CNTF), all members of the IL-6/LIF cytokine family (Cheng et al. 1994; Koshimizu et al. 1996; Hara et al. 1998 and references therein). These factors function through a dimeric transmembrane receptor expressed in PGCs. One subunit of the receptor (e.g., LIF receptor-β) binds specific ligands. The other is a common, non-ligand-binding subunit called glycoprotein 130 (gp130) that acts as a signal transducer by activating STAT-3. Antibodies to gp130 block the activity of LIF on PGCs (Koshimizu et al. 1996). LIF was first tested for its effect on PGCs in vitro because it promotes the undifferentiated, pluripotent phenotype of mouse ES cells in culture; in the absence of LIF and feeder cells, ES cells rapidly differentiate. As described in the previous section, recent studies suggest that LIF functions in combination with a specific level of OCT4 to maintain the undifferentiated phenotype. Signaling through gp130 is therefore likely to play a key role in maintaining the pluripotency and self-renewal ability of PGCs. However, despite the key role apparently played by LIF/gp130 in controlling the PGC phenotype, it is still unclear which member(s) of the ligand family functions in vivo, since mice lacking LIF, LIFRβ, and IL-6 all have normal numbers of PGCs (Koshimizu et al. 1996; Wylie 1999). Likewise, mutation of genes encoding IL-4 and IL-2R has no effect on PGC number even though IL-4 promotes the survival of PGCs in vitro (Cooke et al. 1996). The most likely explanation is that several interleukins regulate PGC proliferation and survival and can compensate for each other in vivo.

Finally, it is very likely that as-yet-unidentified growth factors influence PGC proliferation because optimal growth of the cells in vitro requires fibroblast cell feeder layers. The possible identity of some of these factors has been discussed previously (Bendel-Stenzel et al. 1998).

Although SCF, LIF, and FGF alone have some activity on PGC survival and proliferation in vitro, in combination they have a dramatic effect on the behavior of cells isolated before about 13.5 dpc. Rather than showing a finite number of cell doublings in vitro, the PGCs continue to proliferate indefinitely (Matsui et al. 1992; Resnick et al. 1992; Labosky et al. 1994a). Moreover, the PGCs change their phenotype to resemble pluripotent ES cells that are derived from the inner cell mass cells of the blastocyst. Precisely how this "transdifferentiation" from a PGC to ES cell phenotype is brought about is not known. Like ES cells, embryonic germ-cell-derived cell lines (known as EG cells) can differentiate exten-

sively in culture and can contribute to all the tissues of the embryo, including the germ line, when injected into a host blastocyst (Labosky et al. 1994b; Stewart et al. 1994). However, many undifferentiated EG cells have differences in the methylation of imprinted loci compared with ES cells (Labosky et al. 1994b; Tada et al. 1997). This reflects the fact, discussed in the previous section, that PGCs remove allele-specific parental imprints as they migrate toward, and enter, the gonads. This phenotype is dominant, because if EG cells are fused with somatic cells (thymic lymphocyte), there is demethylation of several imprinted and non-imprinted genes in the somatic nuclei (Tada et al. 1997). It is not yet known whether human EG cell lines show differences in the methylation of imprinted loci (Shamblott et al. 1998).

A process similar to the transdifferentiation of PGCs to EG cells in vitro may occur during the rare in vivo development of teratocarcinomas in the testis of some strains of mice, e.g., 129/Sv. The frequency of testicular teratomas can be increased from about 1% to 95% in the 129/Sv strain by the introduction of the homozygous *Ter* mutation, but the identity of this modifier is not yet known (Asada et al. 1994). Teratomas can also be induced experimentally in vivo in mice by transplanting genital ridges to ectopic sites, or in some rodents from extraembryonic endoderm cells of the early yolk sac (Sobis and Vandeputte 1982). Evidence suggests that this reflects the transdifferentiation of endoderm cells, rather than proliferation of yolk sac cells that have remained undifferentiated.

WHAT CHANGES WHEN PGCs ENTER THE GONAD AND COME INTO CLOSE ASSOCIATION WITH SOMATIC CELLS? THE END OF THE ROAD FOR PGCs

When PGCs enter the genital ridge, they come into close association with somatic gonadal cells derived from the intermediate mesoderm, and after continuing proliferation for a few days, they differentiate into germ cells. By about 13.5 dpc, female PGCs are entering into the prophase of meiosis, while male germ cells go into mitotic arrest and do not resume mitosis until the onset of puberty.

In the mouse, there is a down-regulation of c-KIT receptors in germ cells in the gonad (Manova and Bachvarova 1991). This presumably plays a role in making the germ cells unresponsive to stem cell factor after they have entered the gonad. Since the formation of teratomas is rare, a whole variety of additional mechanisms probably operate normally to protect intragonadal PGCs from the influence of other mitogenic factors. These mechanisms also appear to operate in PGCs that fail to reach the gonad,

since extragonadal teratomas, which are presumed without any direct evidence to be derived from PGCs, are also rare. As discussed earlier, PGCs that come to lie in the fetal adrenal gland cease proliferating and enter meiosis in the mouse (Francavilla and Zamboni 1985). However, experiments in which *Xenopus* PGCs were isolated from the mesentery, labeled in vitro, and then transplanted into the blastoceol cavity of host embryos provided evidence that under these conditions the cells could become incorporated into various tissues and differentiate into somatic cells such as muscle and notochord (Wylie et al. 1985). Analogous experiments in which in-vitro-labeled PGCs from 10.5-dpc mouse embryos were injected into blastocysts failed to show incorporation into either somatic tissues or the germ line (P. Donovan et al., pers. comm.). The ability of individual PGCs to change their fate in ectopic sites in vivo needs to be explored in more detail using robust genetic lineage markers.

In conclusion, PGCs constitute a stem cell population that plays important, evolutionarily conserved roles in germ-line development. The advantages of this population for the organism are that it expands the initially very small pool of germ cell precursors, moves them from extraembryonic regions to the gonads, and helps to ensure that the cells are protected from influences driving them down somatic lineages. The PGCs thus remain pluripotential until they come to the end of the road and differentiate into germ cells.

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REFERENCES

Ambrosetti D.-C., Basilico C., and Dailey L. 1997. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding site. *Mol. Cell. Biol.* **17:** 6321–6329.

Anderson R., Copeland T.K., Scholer H., Heasman J., and Wylie C. 2000. The onset of germ cell migration in the mouse embryo. *Mech. Dev.* **91:** 61–68.

Asada Y., Varnum D.S., Frankel W.N., and Nadeau J.H. 1994. A mutation in the Ter gene causing increased susceptibility to testicular teratomas maps to mouse chromosome 18. *Nat. Genet.* **6:** 363– 368.

Bendel-Stenzel M., Anderson R., Heasman J., and Wylie C. 1998. The origin and migration of primordial germ cells in the mouse. *Semin. Cell Dev. Biol.* **9:** 393–400.

Bendel-Stenzel M.R., Gomperts M., Anderson R., Heasman J., and Wylie C. 2000. The role of cadherins during primordial germ cell migration and early gonad formation in the mouse. *Mech. Dev.* **91:** 143–152.

Braat A.K., Zandbergen T., van de Water S., Goos H.J., and Zivkovic D. 1999. Characterization of zebrafish primordial germ cells: Morphology and early distribution of vasa RNA. *Dev. Dyn.* **216:** 153–167.

Cheng L., Gearing D.P., White L.S., Compton D.L., Schooley K., and Donovan P.J. 1994. Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. *Development* **120:** 3145–3153.

Cooke J.E., Heasman J., and Wylie C.C. 1996. The role of interleukin-4 in the regulation of mouse primordial germ cell numbers. *Dev. Biol.* **174:** 14–21.

Dixon K.E. 1994. Evolutionary aspects of primordial germ cell formation. *Ciba Found. Symp.* **182:** 92–119.

Ephrussi A. and Lehmann R. 1992. Induction of germ cell formation by oskar. *Nature* **358:** 387–392.

Francavilla S. and Zamboni L. 1985. Differentiation of mouse ectopic germinal cells in intra- and perigonadal locations. *J. Exp. Zool.* **233:** 101–109.

Fujiwara Y., Komiya T., Kawabata H., Sato M., Fujimoto H. Furusawa M., and Noce T. 1994. Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila* vasa and its specific expression in germ cell lineage. *Proc. Natl. Acad. Sci.* **91:** 12258–12262.

Ginsberg M. 1994. Primordial germ cell formation in birds. *Ciba Found. Symp.* **182:** 52–67.

Hara T., Tamura K., de Miguel M.P., Mukouyama Y., Kim H., Kogo H., Donovan P.J., and Miyajima A. 1998. Distinct roles of oncostatin M and leukemia inhibitory factor in the development of primordial germ cells and sertoli cells in mice. *Dev. Biol.* **201:** 144–153.

Hubbard E.J. and Greenstein D. 2000. The *Caenorhabditis elegans* gonad: A test tube for cell and developmental biology. *Dev. Dyn.* **218:** 2–22.

Kato Y., Rideout W.M., III, Hilton K., Barton S.C., Tsunoda Y., and Surani M.A. 1999. Developmental potential of mouse primordial germ cells. *Development* **126:** 1823–1832.

Koshimizu U., Taga T., Watanabe M., Saito M., Shirayoshi Y., Kishimoto T., and Nakatsuji N. 1996. Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells. *Development* **122:** 1235–1242.

Labosky P.A., Barlow D.P., and Hogan B.L.M. 1994a. Embryonic germ cell lines and their derivation from mouse primordial germ cells. *Ciba Found. Symp.* **182:** 157–178.

———. 1994b. Mouse embryonic germ (EG) cell lines: Transmission through the germline and differences in the methylation imprint of insulin-growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* **120:** 3197–3204.

Lawson K.A. and Hage W.J. 1994. Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Found. Symp.* **182:** 68–91.

Lawson K.A., Dunn N.R., Roelen B.A., Zeinstra L.M., Davis A.M., Wright C.V., Korving J.P., and Hogan B.L. 1999. Bmp4 is required for the generation of primordial germ

cells in the mouse embryo. *Genes Dev.* **13:** 424–436.

Leatherman J.L., Kaestner K.H., and Jongens T.A. 2000. Identification of a mouse germ cell-less homologue with conserved activity in *Drosophila. Mech. Dev.* **92:** 145–153.

MacGregor G.R., Zambrowicz B.P., and Soriano P. 1995. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development* **121:** 1487–1496.

Manova K. and Bachvarova R.F. 1991. Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. *Dev. Biol.* **146:** 312–324.

Matsui Y., Zsebo K., and Hogan B.L.M. 1992. Derivation of pluripotential embryonic stem cells from murine promordial germ cells in culture. *Cell* **70:** 841–847.

McLaren A. 1994. Germline and soma: Interactions during early mouse development. *Sem. Dev. Biol.* **5:** 43–49.

Monk M., Boubelik M., and Lehnert S. 1987. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99:** 371–382.

Nagano M., Avarbock M.R., Leonida E.B., Brinster C.J., and Brinster R.L. 1998. Culture of mouse spermatogonial stem cells. *Tissue Cell* **30:** 389–397.

Nichols J., Zevnik B., Anastassiadis K., Niwa H., Klewe-Nebenius D., Chambers I., Scholer H., and Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95:** 379–391.

Niwa H., Miyazaki J., and Smith A.G. 2000. Quantitative expression of oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24:** 372–376.

Pesce M., Wang X., Wolgemuth D.J., and Schöler H. 1998. Differential expression of Oct-4 transcription factor during mouse germ cell differentiation. *Mech. Dev.* **71:** 89–98.

Resnick J.L., Bixler L.S., Cheng L., and Donovan P.J. 1992. Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359:** 550–551.

Resnick J.L., Ortiz M., Keller J.R., and Donovan P.J. 1998. Role of fibroblast growth factors and their receptors in mouse primordial gem cell growth. *Biol. Reprod.* **59:** 1224–1229.

Sassone-Corsi P. 1997. Transcriptional checkpoints determining the fate of male germ cells. *Cell* **88:** 163–166.

Seydoux G. and Strome S. 1999. Launching the germline in *Caenorhabditis elegans:* Regulation of gene expression in early germ cells. *Development* **126:** 3275–3283.

Shamblott M.J., Axelman J., Wang S., Bugg S., Littlefield J.W., Donovan P.J., Blumenthal P.D., Huggins G.R., and Gearhart J.D. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci.* **95:** 13726–13731.

Sobis H. and Vandeputte M. 1982. Development of teratomas from yolk sac of genetically sterile embryos. *Dev. Biol.* **92:** 553–556.

Stewart C.L., Gadi I., and Bhatt H. 1994. Stem cells from primordial germ cells can reenter the germ line. *Dev. Biol.* **161:** 626–628.

Tada M., Tada T., Lefebvre L., Barton S., and Surani M. 1997. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* **16:** 6510–6520.

Tam P.P.L. and Snow M.H.L. 1981. Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *J. Embryol. Exp. Morphol.* **64:** 133–147.

Tam P.P.L. and S.X. Zhou 1996. The allocation of epiblast cells to ectodermal and germline lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev. Biol.* **178:** 124–132.

Tam P.P.L., Zhou S.X., and Tan S.-S. 1994. X-chromosome activity of the mouse primordial germ cells revealed by the expression of an X-linked *lac-Z* transgene. *Development* **120:** 2925–2932.

Tan S.-S., Williams E.A., and Tam P.P.L. 1993. X-chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. *Nat. Genet.* **3:** 170–174.

Weidinger G., Wolke U., Koprunner M., Klinger M., and Raz E. 1999. Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. *Development* **126:** 5295–5307.

Wylie C. 1999. Germ cells. *Cell* **96:** 165–174.

Wylie C.C., Heasman J., Snape A., O'Driscoll M., and Holwill S. 1985. Primordial germ cells of *Xenopus laevis* are not irreversibly determined early in development. *Dev. Biol.* **112:** 66–72.

Ying Y., Liu X.-M., Marble A., Lawson K.A., and Zhao G.-Q. 2000. Requirement of Bmp8b for the generation of primordial germ cells in the mouse. *Mol. Endocrinol.* **14:** 1053–1063.

EXHIBIT C

STEM CELL BIOLOGY

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Male Germ-line Stem Cells

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Spermatogenesis is a classic stem cell system. Continuous production of highly differentiated, short-lived sperm is maintained throughout reproductive life by a small, dedicated population of male germ-line stem cells (GSCs). Male GSCs are unipotent, devoted solely to the generation of sperm, much like epidermal stem cells that give rise only to keratinocytes (see Chapter 19). As precursors of the spermatogonial lineage, male GSCs must maintain a balance between the production of mature sperm and the self-renewal of stem cell potential.

Male germ-line stem cells are defined by their function as persistent, clonogenic founders of differentiating germ cells. They can be identified by multiple criteria, including anatomical position within a niche in the testis and distinct behavioral and molecular phenotypes. Male GSCs exhibit many similarities to other stem cell systems. Spermatogonial stem cells are a rare, relatively quiescent population that lies in a protected region in the testis among support cells, which may regulate their behavior. Like all stem cells, male GSCs are the most resistant cells to irradiation or chemical damage. As with hematopoietic stem cells, spermatogonial stem cells in mammals are transplantable, with an ability to both expand the stem cell pool and to regenerate an entire depleted spermatogenic lineage. Spermatogonial stem cells also exhibit signature molecular features, such as high expression of β-1 integrin, much like epidermal and hematopoietic stem cells.

The function of male GSCs has broad implications for development, disease, and evolution. Spermatogenesis is fundamental for the propagation of species. Spermatogenic defects can result in infertility or disease, such as testicular germ cell cancer. The ability to identify, isolate, culture, and alter adult male GSCs will allow powerful new approaches in animal transgenics and human gene therapy relating to infertility and disease. The male

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germ line also offers a powerful experimental system to study fundamental questions in stem cell biology. Spermatogenesis is a well-studied and defined process with many useful tools that can be applied to stem cell research. Current advances such as functional identification of male GSCs, identification of somatic support cells, and tools to characterize their respective roles have established an important foundation for future work.

Spermatogenesis and male fertility, as dependent on the continual production of sperm, have been studied in many organisms ranging from invertebrates to vertebrates (Hannah-Alava 1965). The early germ cell stages of spermatogenesis have been investigated in arthropods (Lindsley and Tokuyasu 1980), moths (King and Akai 1971), teleosts (Upadhyay and Guraya 1973), sharks (Callard et al. 1989), reptiles and amphibians (Pudney 1995), birds (Lin and Jones 1992; Jones and Lin 1993), rodents (Leblond and Clermont 1952a; Oakberg 1956), and primates (Roussel et al. 1969; Clermont 1972; Fouquet and Dadoune 1986). Interesting parallels and contrasts in male GSC behavior have emerged from observations on different organisms. For example, the anatomical location of male GSCs and their intimate association with somatic cells are conserved in many species. One striking difference is variation in the spermatogenic cycle, such as seasonal regulation in species that undergo seasonal breeding compared to continual spermatogenesis throughout reproductive life of non-seasonal breeders.

This chapter focuses on male germ-line stem cell biology in the fruit fly *Drosophila melanogaster* and in mammals (primarily rodents). Much of the information on male GSC identification and function has resulted from the helpful tools and methods available in these systems. There are striking similarities in male GSC location, behavior, and function between *Drosophila* and mammals, suggesting that complementary approaches in the two systems will help illuminate the mechanisms of male GSC regulation.

Drosophila provides a genetic system for rapid identification of genes required for normal male GSC behavior based on unbiased mutagenesis screens (for review, see Fuller 1993). In addition, tools for mitotic recombination and genetic marking (Xu and Rubin 1993; Lee and Luo 1999) can be used in lineage analysis experiments to identify and follow the behavior of male GSCs, either in the wild type or in mutants (Gönczy and DiNardo 1996). Similar mitotic recombination tools allow tests of whether a gene required for normal stem cell behavior functions cell-autonomously (in the germ line) or non-autonomously (in the soma) (Gönczy et al. 1997; Matunis et al. 1997; Kiger et al. 2000; Tran et al. 2000). Finally, the transparency and simple organization of the *Drosophila* testis allow in vivo identification of spermatogonial stem

cells and phenotypic demonstration of mutational effects without disrupting the local stem cell microenvironment.

In mammals, early spermatogonial cells, including the GSC population, can be physically manipulated. Donor male GSCs can be transplanted into recipient host testes to assay stem cell function (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). The transplant assay can be used for lineage analysis or to test whether requirement for a gene function is cell-autonomous or non-cell-autonomous (Ogawa et al. 2000). As in the fly, the ability to dissect and section the mammalian testis allows phenotypic analysis of spermatogonial cells in situ. In addition, the ability to make targeted gene disruptions in mice can be used to test the genetic requirements for male GSC function.

Many of the questions that arise about male GSC biology could be asked of stem cells in any system. What regulates male GSC specification from precursor populations in the embryo? What regulates initiation of the spermatogonial stem cell divisions? How are male GSCs defined and identified? Where are GSCs located in the testis, and what cells make up their microenvironment? Do somatic cells form a niche that regulates GSC behavior? Are stem cell divisions asymmetric or symmetric? What are the molecular mechanisms that maintain a balance between self-renewal of stem cell identity and initiation of differentiation when stem cells divide? Are these critical mechanisms under intrinsic or extrinsic control?

We review these questions below, each with an introductory discussion on male GSCs in general, followed by more detailed information that specifically relates to *Drosophila* or mammalian male GSCs. For more information, see earlier reviews on germ-line stem cells (Cooper 1950; Hannah-Alava 1965; Lindsley and Tokuyasu 1980; Fuller 1993; Meistrich and van Beek 1993a; de Rooij and Grootegoed 1998; Lin 1998).

SPECIFICATION OF MALE GERM LINE AND INITIATION OF STEM CELL DIVISIONS

The male germ line proceeds through several developmental steps prior to establishment and initiation of spermatogonial stem cell divisions in the testis (Fig. 1) (for detailed description, see Pringle and Page 1997; Saffman and Lasko 1999; Wylie 1999). Primordial germ cells (PGCs) are specified as distinct from somatic cell lineages at one of the earliest stages in embryogenesis (Fig. 1a). The PGCs proliferate and migrate from their site of origin to the future position of the gonad (Fig. 1b), where they associate with somatic gonadal precursor cells to form the gonad (Fig. 1c). Once within the gonad, the PGCs differentiate in a sex-specific manner, including a distinct program of proliferation and quiescence (Fig. 1d,e).

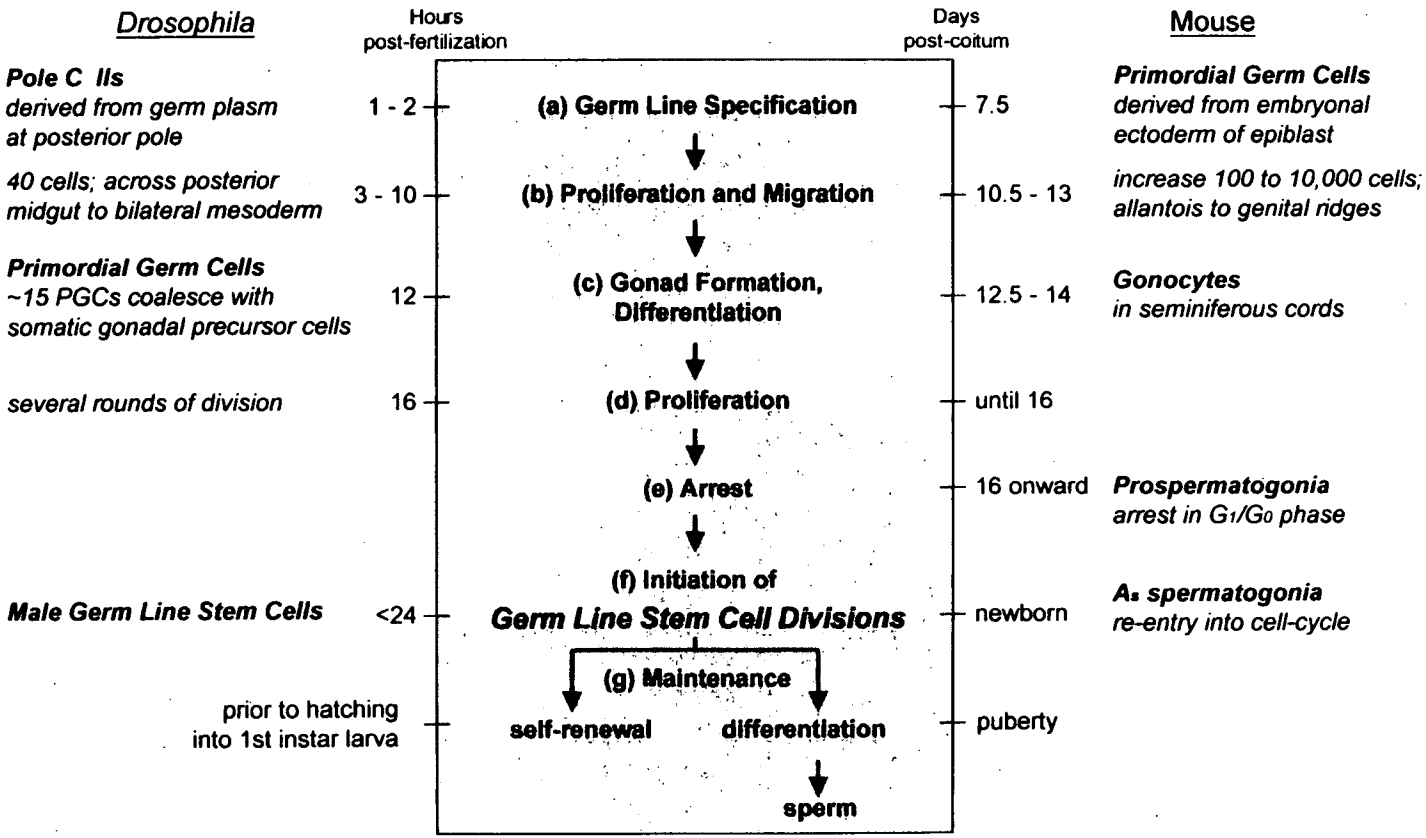

Figure 1 Specification and initiation of male germ-line stem cell divisions.

PGCs in the testis become male germ-line stem cells and initiate the first round of spermatogenesis that will produce sperm at the onset of reproductive age (Fig. 1f). Finally, male germ-line stem cell divisions are regulated so that appropriate numbers of stem cells are maintained (Fig. 1g).

When does the germ line first acquire stem cell potential? Evidence in mammals suggests that the earliest PGCs derived in the embryo already exhibit aspects of stem cell potential under certain experimental conditions. Unlike spermatogonial stem cells, however, PGCs are pluripotent, giving rise to highly differentiated cells of multiple somatic and germ cell lineages (Matsui et al. 1992; Resnick et al. 1992; Shamblott et al. 1998). In both flies and mammals, PGCs display additional characteristic differences from germ-line stem cells established in the adult male. PGCs can proliferate expansively with no differentiation. In contrast, under normal conditions, germ-line stem cells maintain their numbers and give rise to differentiating gametes. PGCs migrate throughout the embryo, whereas germ-line stem cells normally stay within a well-defined niche. Finally, PGCs are similar in appearance and behavior in both sexes, whereas germ-line stem cells are normally restricted to either male or female gametogenesis, the products of which exhibit extreme sexual dimorphism.

Environmental cues may direct the transition from PGC to male germ-line stem cell after populating the embryonic gonad. Somatic gonadal cells are required for proper development of male germ cells, including expression of sex- and stage-specific genes (Staab et al. 1996). The somatic gonad also appears to direct the sex-specific program and the timing of onset of gametogenesis (for review, see Pringle and Page 1997). However, the somatic gonadal cells do not require the presence of germ cells for testis formation (Geigy 1931; Aboim 1945; Mintz and Russell 1957).

The establishment and long-term maintenance of male germ-line stem cell divisions appear to be regulated independently. Only a subpopulation of the PGCs is maintained as male germ-line stem cells through adulthood (Hardy et al. 1979; de Rooij 1998). One hypothesis is that only some PGCs acquire and/or retain stem cell capacity, whereas other PGCs directly differentiate without self-renewing divisions. Although the mechanisms are not understood, it is thought that signals from the somatic gonad direct which cells from an initially uniform PGC population are retained and which cells differentiate.

Fly

Germ-line specification and migration in the early *Drosophila* embryo have been studied extensively (for recent reiews, see Rongo et al. 1997;

Saffman and Lasko 1999). Germ cells are the first cells formed when pole cells bud off at the posterior end of the syncytial embryo (Fig. 1a). The pole cells proliferate to yield a final population of up to 40 germ cells (Sonnenblick 1941). To reach the developing gonad, the pole cells are first passively moved with the posterior midgut invagination, then actively migrate across the midgut to contact the mesoderm (Fig. 1b). The pole cells split into two groups, migrate into bilateral clusters, and interact with a well-characterized population of somatic gonadal precursor cells (Boyle and DiNardo 1995; Boyle et al. 1997; Moore et al. 1998). Approximately 15 PGCs and 30 somatic gonadal precursor cells coalesce to form the embryonic male gonad (Sonnenblick 1941; Poirie et al. 1995). After gonad formation, the pole cells are called primordial germ cells in *Drosophila* (Fig. 1c) (Sonnenblick 1941), whereas in mammals germ cells are usually referred to as gonocytes at this stage.

Much less is known about the transition from primordial germ cell fate into male germ-line stem cell identity in *Drosophila*. Early germ cells exhibit sex-specific differences once inside the embryonic gonad. For example, a certain germ cell marker expressed in the male embryonic gonad is not expressed in females (Staab et al. 1996). Gametogenesis is also initiated at distinct times in males versus females. Male germ cell proliferation and initiation of spermatogenesis must take place prior to hatching of the embryo, because differentiating spermatogonia are found in first-instar larval testes (Fig. 1d,f) (Kerkis 1933; Sonnenblick 1941). In contrast, female germ cells do not initiate oogenesis until days later, at the time of the larval–pupal transition (King 1970). Since male GSCs are active throughout larval and adult life, stem cell activity can be assayed in larval testes, independent of adult male viability or fertility. This is helpful when studying genes required for both adult viability and stem cell behavior (see last section). From the initial ~15 PGCs in the embryonic testis, only 5–9 male germ-line stem cells are maintained in the adult testis (Fig. 1g) (Hardy et al. 1979).

Mammals

Primordial germ cells are induced in the embryonic ectoderm of the mammalian epiblast (Fig. 1a) (Everett 1943; Lawson et al. 1999; Chapter 9). In the mouse embryo at 7 days, approximately 100 PGCs are detected in the extraembryonic mesoderm based on alkaline phosphatase activity, a marker of germ cell identity (Ginsburg et al. 1990). The PGCs then migrate from the allantois along the hindgut to the genital ridges

(Saffman and Lasko 1999). PGCs proliferate during their migration (Fig. 1b), with more than 10,000 PGCs eventually populating the genital ridges (Tam and Snow 1981).

In males, PGCs are enclosed in seminiferous cords to form the gonads. The somatic environment in the seminiferous tubules triggers the male-specific differentiation of PGCs into gonocytes (Clermont and Perey 1957; Pringle and Page 1997). Gonocytes are morphologically distinct from either the PGCs or differentiating female germ cells. Gonocyte identity is marked by transition into a nonmitotic state, growth in cell size, and the onset of distinct gene expression (Huckins 1963; Li and Gudas 1997). It is hypothesized that somatic Sertoli cells in the seminiferous tubules produce an inhibitory signal that prevents differentiation of PGCs into female germ cells and allows progression along the male pathway (for review, see Pringle and Page 1997). Cessation of PGC proliferation and gonocyte survival are also dependent on the somatic cells. Whereas PGCs survived in vitro when cocultured with one of any multiple somatic cell types, gonocytes survived only when cocultured in the presence of Sertoli cells (Resnick et al. 1992; van Dissel-Emiliani et al. 1993).

Gonocytes relocate to the basal lamina during morphogenesis of seminiferous somatic cells (Orth 1993). Gonocytes undergo subsequent proliferative expansion, producing up to 50,000 cells in the mouse, then mitotically arrest in the G_1/G_0 phase until after birth (Saffman and Lasko 1999). Stem cells may develop directly from gonocytes (Clermont and Perey 1957) or may arise indirectly via prospermatogonia that differentiate at the time of the gonocyte mitotic arrest (Hilscher et al. 1974). In the newborn mouse, spermatogonial stem cells are likely to arise when proliferation resumes. Finally, onset of puberty triggers initiation of differentiation along the spermatogenic lineage. In mammals, as in the fly, it is difficult to conclude precisely when male germ-line stem cell identity is established. Some interconnected gonocytes have been found in the embryonic gonad, which may reflect initiation of spermatogenesis as early as in the gonocyte stage (Zamboni and Merchant 1973). In rat and hamster, kinetic studies indicate that some gonocytes may differentiate directly into spermatogonia (van Haaster and de Rooij 1994), suggesting that only a subpopulation of gonocytes retain stem cell status.

ROLE OF GERM-LINE STEM CELLS IN SPERMATOGENESIS

Male germ-line stem cells must self-renew as well as produce progeny that initiate differentiation. To study this crucial aspect of stem cell behav-

ior and to unambiguously identify GSCs, we need to understand the relationship of spermatogonial stem cells to the more differentiated germ cells that comprise the spermatogenic lineage.

As in other stem cell systems, differentiation along the spermatogenic lineage progresses through three distinct compartments (Fig. 2) (Loeffler and Potten 1997; Fuchs and Segre 2000). Male GSCs maintain the lineage throughout reproductive age by balancing stem cell self-renewal with differentiation (Fig. 2, I). This balance can be obtained either by asymmetric or symmetric stem cell divisions (discussed in detail below). Stem cell daughter cells that initiate differentiation undergo several rounds of mitotic amplification divisions (Fig. 2, II). These mitotic divisions are specialized in that cytokinesis is incomplete, resulting in cysts of synchronously dividing, interconnected germ cells (Phillips 1970; Dym and Fawcett 1971). Due to the amplification divisions, a single daughter cell commit-

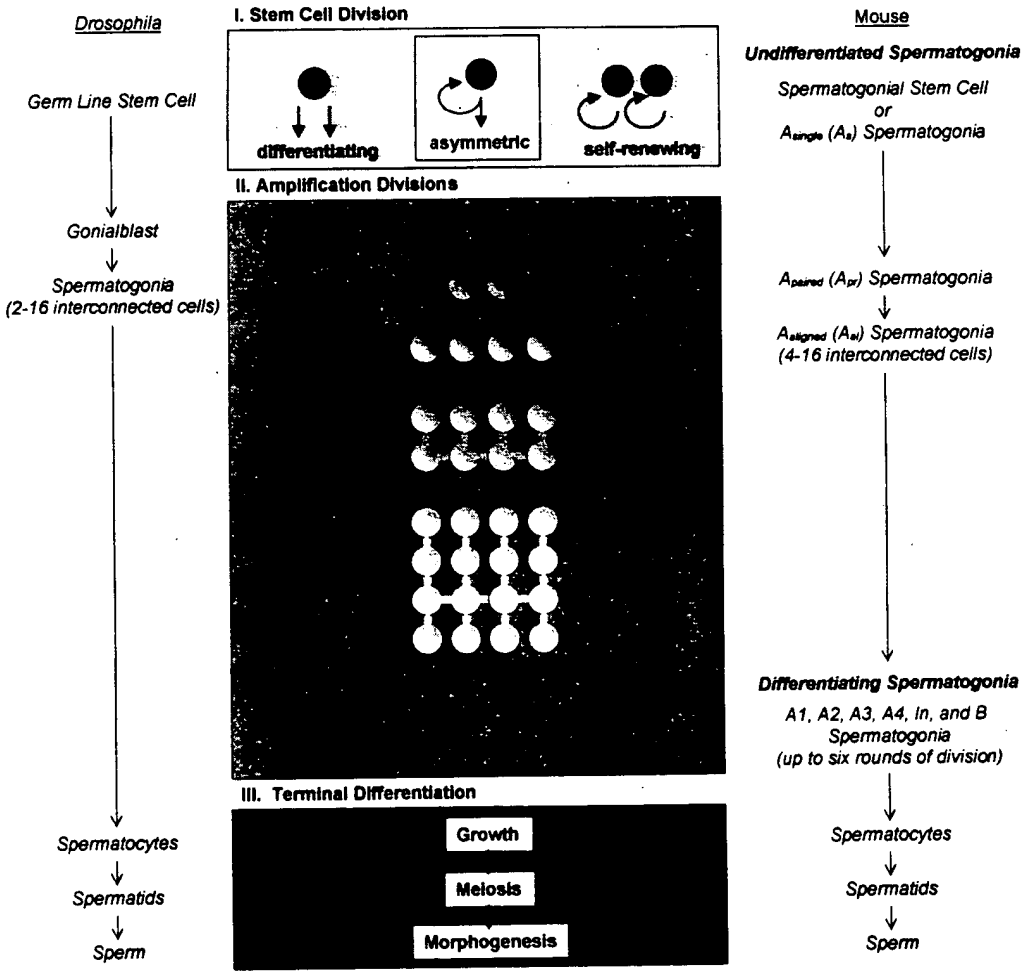

Figure 2 Male germ-line stem cells in the spermatogenic lineage.

ted to differentiation eventually gives rise to large numbers of sperm. In this regard, germ cells in the amplification divisions are comparable to progenitor cells in the hematopoietic lineage, which maintain a limited proliferative capacity (Weissman 2000). Finally, the germ cells exit from mitotic division and initiate terminal differentiation (Fig. 2, III), including progression through the stages of spermatocyte growth, the meiotic program, and morphogenesis into spermatozoa. The entire process of spermatogenesis takes approximately 10 days in *D. melanogaster*, 35 days in mice, and 61 days in rats (Clermont and Trott 1969; Lindsley and Tokuyasu 1980; Meistrich and van Beek 1993b).

Spermatogenesis is a complex process that is regulated at multiple stages. Thus, infertility is not necessarily due to a defect in the stem cell compartment. However, it has been suggested for mammals that perturbations in later stages may affect or alter stem cell behavior, indicating feedback control on the GSC population (Huckins and Oakberg 1978).

Fly

In *Drosophila*, male GSC divisions normally have an asymmetric outcome (Gönczy and DiNardo 1996). One daughter cell self-renews stem cell identity and one daughter cell initiates differentiation as a gonialblast (Fig. 2). The gonialblast is the founder spermatogonial cell that initiates the spermatogonial amplification divisions, much as the cystoblast in the *Drosophila* female germ line initiates the amplification divisions that produce interconnected germ cells of the developing egg chamber (Lin 1997). In *D. melanogaster*, precisely four rounds of synchronous amplification division invariably result in 16 interconnected spermatogonia (Tihen 1946). The number of amplification divisions varies among different *Drosophila* species, suggesting that germ cell proliferation is restricted at a distinct regulatory point that is under genetic control (Fuller 1993).

Stem cells divide asynchronously and continuously throughout larval and adult life (Cooper 1950; Hardy et al. 1979; Lindsley and Tokuyasu 1980; Gönczy and DiNardo 1996). Continuous stem cell activity ensures ongoing replenishment of gonialblasts to initiate differentiation, with the effect that all stages of spermatogenesis are present in the testis at any one time.

Mammals

In mammals, all premeiotic male germ cells, including stem cells, are called spermatogonia. The different stages of spermatogonia are roughly classified by temporal order as either undifferentiated A-type spermato-

gonia or differentiating spermatogonia (Leblond and Clermont 1952b; Oakberg 1956; Huckins 1971b). GSCs are among the single undifferentiated A spermatogonia, termed A_{single} (A_s) spermatogonia. A_s spermatogonia self-renew stem cell identity and give rise to other undifferentiated A-type spermatogonia that eventually differentiate. The successive stages of undifferentiated spermatogonia proliferate synchronously as A_{paired} (A_{pr}) spermatogonia in clusters of 2 germ cells and $A_{aligned}$ (A_{al}) spermatogonia in clusters of 4, 8, or 16 germ cells (Fig. 2). Undifferentiated A spermatogonia develop into differentiating spermatogonia, which continue through six additional rounds of synchronous interconnected division as A_1, A_2, A_3, A_4, Intermediate, and finally, B spermatogonia (Fig. 2). Within a given region of the seminiferous tubules, mammalian spermatogenesis is cyclic, with synchronous bursts in stem cell activity alternating with periods of relative inactivity (Leblond and Clermont 1952b; Oakberg 1956) (see below).

LOCATION OF MALE GERM-LINE STEM CELLS IN THE TESTIS

Male germ-line stem cells are localized anatomically to a defined compartment within the testis. The ability to identify stem cells in situ is at present relatively unique to the male germ line, in contrast to the difficulty of unambiguously identifying most other stem cell types in vivo (e.g., hematopoietic stem cells within the bone marrow). Spermatogenesis proceeds in a spatial gradient within the testis (Figs. 3 and 4), allowing GSC activity to be easily visualized as the continual production of differentiating germ cells.

Fly

The *Drosophila* adult testis is a coiled tube closed at the apical end and opening at the base into the seminal vesicle (Fig. 3a). The progression of spermatogenesis transits the length of the tube. The GSCs reside in the germinal proliferation center at the apical testis tip (Fig. 3b,c; S) (Hardy et al. 1979). Just distal to the stem cells lie the gonialblasts and interconnected spermatogonia. Cysts of 16 growing primary spermatocytes are displaced down the testis tube, completing meiosis approximately one-third of the way toward the base. Elongating spermatids extend back up the length of the testis lumen (Fig. 3a, arrowheads) then exit the basal

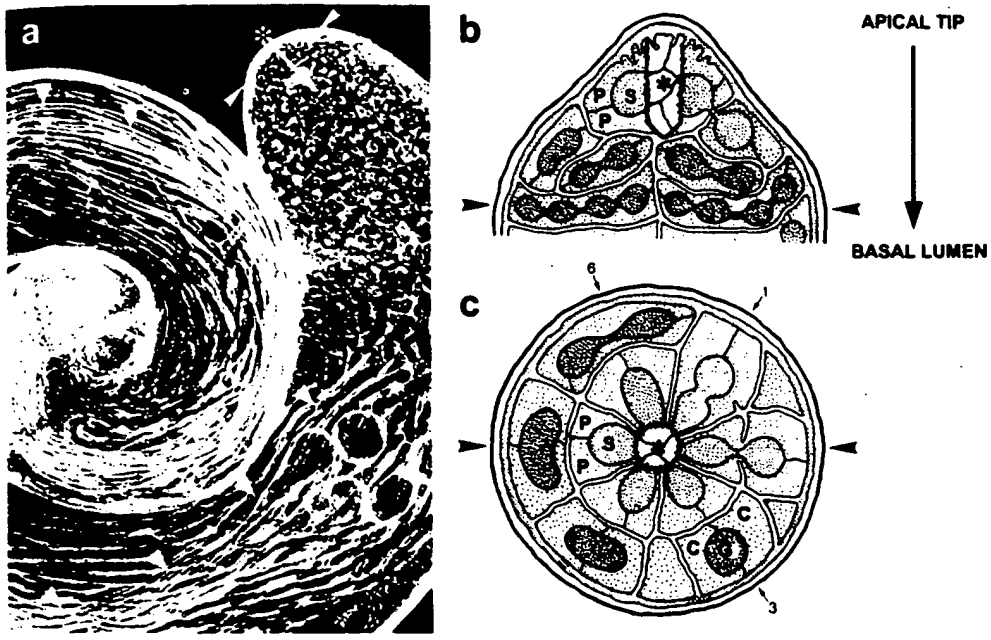

Figure 3 Location of male germ-line stem cells in the *Drosophila* testis. (*a*) The *Drosophila* adult testis is a coiled tube closed at the apical end (*asterisk*). All germ cell stages of spermatogenesis are visible through the transparent testis wall when viewed by phase microscopy. GSCs reside at the apical testis tip within the germinal proliferation center (*asterisk*). Successive stages of germ cell differentiation are displaced basally (*arrow*), with elongated sperm extending back up the testis (*arrowheads*). Schematic illustrations of (*b*) a transverse section and (*c*) a cross section through the germinal proliferation center of the testis apical tip. Asterisks and shaped arrowheads correspond approximately to those in *a*. Arrows labeled *1*, *3*, and *6* denote successive stages in early spermatogenesis: (1) stem cell division, (3) formation of a differentiating germ-line cyst, and (6) initiation of spermatogonial divisions. Spermatogenesis proceeds from the apical tip to the base of the lumenal tube (*vertical arrow*). Both the GSCs (*light blue, S*) and the somatic cyst progenitor cells (*yellow, P*) divide radially and asymmetrically (*arrow 1*) around the hub (*green, asterisk*). Note that the GSCs and cyst progenitor cells normally do not divide in synchrony as drawn. The GSC daughter cell adjacent to the hub self-renews stem cell identity, whereas the daughter farthest from the hub initiates differentiation as a gonialblast (*dark blue, G*). Similarly, the cyst progenitor cells divide to give rise to one cyst progenitor cell and one cyst cell committed to differentiation (*orange, C*). One gonialblast is enclosed in two cyst cells to form a germ-line cyst (*arrow 3*), which proceeds through spermatogonial divisions (*arrow 6*). A basement membrane overlays the hub and stem cells (*gray stippled*). The testis is enclosed in a bilayer sheath made up of muscle and pigment cells (*white*). (*b*, *c*, Modified, with permission, from Lindsley and Tokuyasu 1980 [© Academic Press].)

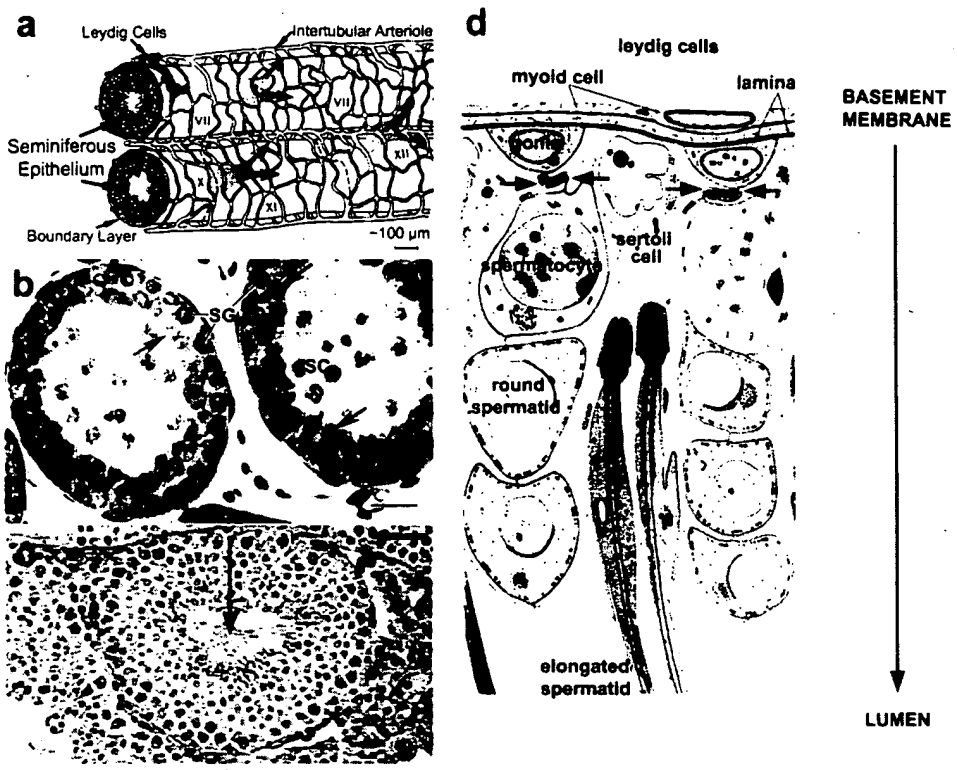

Figure 4 Location of male germ-line stem cells in the mammalian testis. (*a*) Schematic of just two seminiferous tubules from a mammalian testis. Spermatogenesis takes place in the seminiferous epithelium of the tubule lumens. Somatic boundary cells surround the seminiferous epithelium. The vasculature and somatic Leydig cells reside in the interstitial space that surrounds each tubule. Successive stages of the epithelial cycle are found along the length of the tubules, which are defined by the representative germ cell contents (Roman numerals). (Modified, with permission, from Hinton and Turner 1993 [© Oxford University Press].) (*b*) Autoradiograph of a cross-section containing two seminiferous tubules from a 21-day-old rat testis. The spermatogonia (SG) are mitotically active and incorporate ^{3}H[thymidine] at the lumen periphery. Spermatocytes (SC) are postmitotic and localize further within the lumen. Somatic Sertoli cells (*arrows*) neighbor the spermatogonia. (Modified, with permission, from Orth 1982 [©Wiley].) (*c*) Cross-section of one seminiferous tubule from an adult mouse testis stained with hematoxylin and eosin. Spermatogenesis proceeds radially inward (*arrow*), from spermatogonia at the lumenal edge to sperm in the central lumen. (Modified, with permission, from Ogawa et al. 2000.) (*d*) Schematic of a seminiferous epithelium. Spermatogenesis proceeds radially from the basement membrane to the inner lumenal space (*vertical arrow*). Spermatogonia reside adjacent to the basement membrane surrounded by somatic myoid cells. Differentiating germ cells move inward, progressing through the spermatocyte and spermatid stages. Somatic Sertoli cells flank germ cells of all stages, and form a special junction enclosing spermatogonia into an exterior, basal compartment (*small arrows*). (Modified, with permission, from Hecht 1993. [© Kluwer Academic/Plenum].)

opening (Lindsley and Tokuyasu 1980; Fuller 1993). A similar spatial gradient is observed in larval testes, with the difference that the larval testis is ovoid and normally contains only premeiotic germ cells and spermatocytes (Kerkis 1933; Sonnenblick 1941). The germ cells at the apical testis tip can be visualized by phase microscopy of live dissected testes, although the stem cells and spermatogonia are difficult to distinguish without the aid of molecular markers.

Mammals

Mammalian spermatogenesis takes place in the seminiferous epithelium, inside the multiple seminiferous tubules that compose the testis (Fig. 4a). Male GSCs lie at the tubule periphery next to the basement membrane (Hadley and Dym 1987). Germ cells move radially inward as spermatogenesis proceeds until the sperm are released into the central lumen (Fig. 4b,c). Spermatogenesis occurs in successive waves along the length of the tubules (Fig. 4a). Each wave contains a discrete cohort of germ cell stages, categorized as either 12 epithelial stages in the mouse (I–XII) or 14 stages in the rat (I–XIV) (Leblond and Clermont 1952b; Oakberg 1956). In the mouse, for example, undifferentiated spermatogonia normally divide during stages X–III, but rarely divide during the other stages (Oakberg 1971). The dynamics of the epithelial cycle imply that stem cell activity is selectively reinitiated and/or inhibited within groups of staged spermatogonia.

THE STEM CELL NICHE: THE RELATIONSHIP BETWEEN GERM-LINE STEM CELLS AND THE SOMA

Male germ-line stem cells are closely associated with somatic cells. Throughout the testis, the local microenvironment varies along with the different spermatogenic stages. Male GSCs lie within a protected region of the testis, surrounded by somatic cells that isolate them from differentiating germ cells. The close association between GSCs and specific somatic cell types or stages suggests a role for somatic support cells in regulating GSC behavior.

Fly

Drosophila male germ-line stem cells are associated with two somatic cell types within the germinal proliferation center (Figs. 3b,c and 5a,d) (Aboim 1945; Smith and Dougherty 1976; Hardy et al. 1979). GSCs lie in a ring closely apposed to a cluster of somatic apical cells (Fig. 5c) that

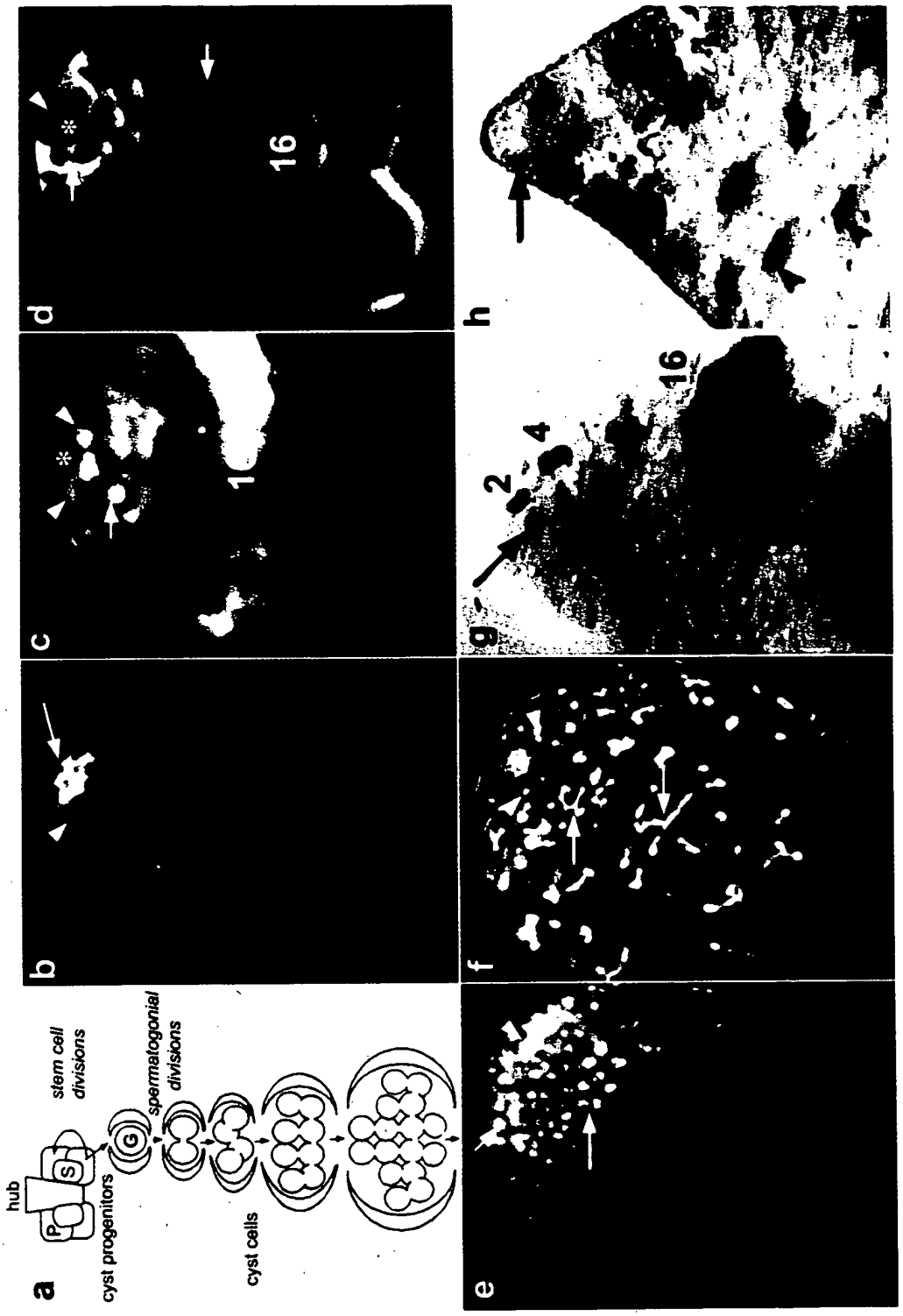
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Figure 5 Identification of *Drosophila* male germ-line stem cells in the niche. (*a*) Schematic of the *Drosophila* germinal proliferation center. The GSCs (*light blue*) and somatic cyst progenitor cells (*yellow*) form a ring around the somatic hub (*green*). The germ-line gonialblast (*blue*) is encysted by two cyst cells (*orange*), then initiates the spermatogonial divisions. (*b*) The hub at the apical tip can be detected by immunofluorescence using antibodies against epithelial cell markers such as *Drosophila* E-cadherin (*arrow*). Background staining of testis was shown to help visualize the testis and is not specific to E-cadherin. The ring of GSCs that surround the hub are not visible (*arrowhead*). (*c*) The germ cells in the germinal proliferation center as detected by tissue-specific expression of the green fluorescent protein (GFP). A ring of GFP-positive, single, GSCs (*arrowheads*) surrounds the unlabeled hub (*asterisk*). Single germ cells displaced from the apex are presumptive gonialblasts formed from radial stem cell divisions (*arrow*). Interconnected germ cells in the spermatogonial stages are distinguished as clusters of 2, 4, 8, and 16 cells with GFP throughout a shared cytoplasm. GFP expression under control of the *nanos-GAL4, UAS-GFP* transgenes is down-regulated during the spermatocyte growth phase. (*d*) The somatic cyst cells in the germinal proliferation center as detected by tissue-specific expression of GFP. The cyst cell cytoplasm is thin and lacy, stretching around the germ cells. The cyst progenitor cells (*arrow at tip*) contact the hub (*asterisk*) and enclose the round, unlabeled GSCs (*arrowhead*). Cyst cells (*arrow*) continue to grow and enclose the differentiating germ cells that increase in size and number. GFP expression is via the transgenes *ptc-GAL4, UAS-GFP*. (*e*) Division patterns distinguish stem cells and gonialblasts from spermatogonia by detection of the mitotic indicator, phosphorylated Histone 3 (*green*), and DNA (*purple*). The GSCs and gonialblasts divide asynchronously as single cells (*arrowhead*), and are normally only found near the hub (*asterisk*). Further from the hub but still within the apical tip, the interconnected spermatogonia undergo synchronous amplification divisions as groups of 2, 4 (*short arrow*), or 8 (*long arrow*) cells. At the 16-cell stage, spermatogonia exit mitosis. (*f*) Fusome structure distinguishes stem cells and gonialblasts from spermatogonia by immunofluorescence detection of components such as α-Spectrin. A ball-shaped fusome, or spectrosome (*arrowheads*), is only found adjacent to the hub (*asterisk*) in GSCs and gonialblasts. Branched fusome passes through the ring canals of interconnected clusters of 2-, 4-, 8-, and 16-cell spermatogonia and spermatocytes (*arrows*). (*g*, *h*) Testes analyzed 5 days after heat-shock-induced mitotic recombination and activation of the *lacZ* nuclear lineage marker. (Modified, with permission, from Gönczy and DiNardo 1996) [© Company of Biologists Ltd.].) (*g*) Lineage tracing of a persistent GSC clone using a nuclear marker. A GSC is marked as a single, dividing cell (*arrow*) adjacent to the hub (*unlabeled*). Successive progeny derived from the marked GSC are seen as more mature spermatogonial (2, 4) and spermatocyte (16) clusters. (*h*) Lineage tracing of a persistent somatic cyst cell clone using a nuclear marker. A cyst progenitor cell is marked as a single cell (*arrow*) near the testis apex. Progeny derived from the marked cyst progenitor cell are detected as marked cyst cell nuclei (*arrowheads*) associated with packets of developing germ cells.

form a compact structure called the hub (Hardy et al. 1979). The apical cells of the hub exhibit epithelial-like characteristics, such as localization of fasciclin III (Brower et al. 1981) and E-cadherin at the cell membrane (Fig. 5b). A pair of somatic stem cells, the cyst progenitor cells, flanks each germ-line stem cell. The cyst progenitor cells contact the hub with cytoplasmic extensions, thus enclosing the GSCs (Figs. 3b,c, and 5d) (Hardy et al. 1979). The development of hub and cyst cells does not require the presence of the germ line (Geigy 1931; Aboim 1945), supporting their somatic origin. Like the GSCs, cyst progenitor cells divide asymmetrically to self-renew a cyst progenitor cell and to produce a cyst cell that initiates differentiation (Hardy et al. 1979; Gönczy and DiNardo 1996). Two somatic cyst cells enclose one germ-line-derived gonialblast to form a unit called a germ-line cyst (Stern 1941). The two cyst cells do not divide again, but continue to enclose the growing clone of differentiating germ cells (Figs. 3b,c, and 5d). Germ cells and somatic cyst cells are easily distinguished from one another on the basis of cell morphology and marker expression.

The testis is enclosed in a sheath made up of an inner layer of muscle cells and an outer layer of squamous, pigmented cells (Geigy 1931; Stern 1941). The muscle layer of the sheath is open only over the apical cells of the hub (Hardy et al. 1979). The germinal proliferation center, however, is firmly attached to the testis apex by a thick, convoluted layer of basal lamina that overlays the hub (Fig. 3b) (Hardy et al. 1979). If the sheath is torn open from a dissected testis, cysts of differentiating germ cells spill freely away from the testis wall, while the germ cells in the germinal proliferation center remain tightly associated with the testis apex.

The number of GSCs appears to be developmentally regulated. Morphological studies by reconstruction from serial electron micrographs indicated that the number of GSCs decreases from 16–18 cells in third-instar larval testes to 5–9 cells in 3-day-old adult testes (Hardy et al. 1979). Similarly, cyst progenitor cell numbers also decrease from 19–20 cells in larval stages to 9–17 cells in the adult, with two cyst progenitor cells associated with every GSC. Concomitantly, the size and shape of the hub is compacted from a sheet of 16–18 cells into a densely packed, dome-shaped structure of 8–16 cells in the adult. It is possible that only the germ cells that maintain contact with the developing somatic hub retain GSC identity.

Mammals

Mammalian spermatogonial stem cells are neighbors to several somatic cell types along the basement membrane of the seminiferous tubule (Fig. 4d). The most closely apposed somatic cells are the large Sertoli cells.

Sertoli cells contact all stages of developing germ cells, extending processes from the basement membrane to within the central lumen of the epithelium (Russell et al. 1983). The Sertoli cells create distinct microenvironments along their length via directional secretion of multiple regulatory factors (Bardin et al. 1993). Sertoli cells form a tight junction barrier that isolates the spermatogonia into an exterior or basal compartment of the epithelium, separating them from the more differentiated germ cell stages within the interior or lumenal compartment (Fig. 4d, small arrows) (Dym and Fawcett 1970; Gilula et al. 1976). This barrier may serve to expose and/or shield stem cells from specific regulatory signals (Bardin et al. 1993). GSCs may also form junctions directly with the Sertoli cells. Desmosomes formed between spermatogonia and Sertoli cells have been described in vivo (Russell et al. 1983).

Peritubular myoid cells and the lymphatic endothelium together form a somatic boundary layer that encloses the basement membrane surrounding the spermatogonial stem cells. The myoid cells, along with Sertoli cells, secrete the underlying basement membrane as well as regulatory factors that affect Sertoli cell behavior (Skinner et al. 1985). Although the somatic Leydig cells in the intertubular spaces do not directly contact the spermatogonial stem cells, they do secrete steroids and peptides with potential regulatory roles in spermatogenesis (Ewing and Keeney 1993).

IDENTIFICATION OF MALE GERM-LINE STEM CELLS BY PHENOTYPE AND FUNCTION

Male germ-line stem cells can be distinguished from other early germ cells by distinct behavioral and molecular phenotypes. Generally, male GSCs exhibit an asynchronous pattern of mitotic division and a slower cell cycle time, making them more resistant to damage from low doses of radiation than the later undifferentiated or differentiated spermatogonia (Dym and Clermont 1970). Since the location of male germ-line stem cells within the testis is known, specific gene products can be assayed for expression in either the GSCs or their differentiated progeny. Investigation of the function of genes that are expressed in stem cells but not in more differentiated spermatogonia, or vice versa, may help uncover intrinsic mechanisms that specify stem cell self-renewal versus differentiation. In addition, such cell-type-specific expression markers greatly facilitate the analysis and interpretation of mutant phenotypes.

Stem cells are most stringently defined by their functional capacity. To be classified as a stem cell by this definition, a single, adult, male germ

cell must demonstrate the ability to self-renew stem cell identity and continually produce cells that differentiate into sperm. Methods to identify male GSCs functionally have been developed for both the fly and mammalian systems.

Fly

In *Drosophila*, a combination of expression markers, characteristic subcellular structures, and division behavior are used to distinguish stem cells from later germ cell stages. In the wild-type testis, all cells undergoing mitotic division are restricted to the region of the apical tip (Figs. 3 and 5e). Stem cells and gonialblasts are distinguished from spermatogonia as the single cells that divide asynchronously close to the hub (Tihen 1946; Hardy et al. 1979; Gönczy and DiNardo 1996). In contrast, interconnected spermatogonia divide in synchrony and normally lie at a greater distance from the hub (Fig. 5e, arrows). A stem cell division was calculated to take place once every 10 hours at the apical tip of an adult testis (Lindsley and Tokuyasu 1980). Only one GSC in mitosis was observed in 50 GSCs examined morphologically (Hardy et al. 1979).

Drosophila germ cells have a characteristic subcellular structure called the fusome, which is composed of multiple membranous and cytoskeletal components, including α-spectrin (Lin et al. 1994). The fusome takes on a different shape in single germ cells and in interconnected germ cells. In GSCs and gonialblasts, this subcellular structure forms a ball-shaped spectrosome (Fig. 5f, arrowheads). Spectrosomes are normally found in a rosette of cells only one or two cells deep from the apical hub. In contrast, the fusome is extended and branched, running through the ring canals that connect mitotically related spermatogonia or spermatocytes within a cyst (Fig. 5f, arrows).

Distinct gene expression profiles can further define stem cell versus spermatogonial identities. A collection of enhancer trap lines was isolated based on distinct expression patterns of the *lacZ* marker gene in testes (Gönczy et al. 1992; Gönczy 1995). For example, the enhancer trap markers *M34a* and *S1-33* drive expression of β-galactosidase activity in the GSCs and gonialblasts at the most apical tip, but not in later stages (Gönczy 1995). Other lines mark different stages of differentiating germ cells or the accompanying somatic cyst cells. Antibodies to specific proteins also detect subpopulations of germ cells. The cytoplasmic *bag-of-marbles* protein (Bam-C) is detected in spermatogonia from the 2- to 16-cell stages, but not in the ring of germ-line stem cells around the hub (McKearin and Ohlstein 1995; Gönczy et al. 1997).

Clonal Analysis as a Test of Drosophila *Male Germ-line Stem Cell Function*

Male germ-line stem cells are genetically identified in the *Drosophila* testis by clonal analysis. The existence of GSCs was first proposed in 1929 to explain the size and distribution of clusters of identical mutations in brooding studies (Harris 1929). Analysis of the progeny from irradiated males demonstrated that large groups of identical mutant progeny must have derived from the division of one original, mutagenized germ cell. On the basis of these studies, it was proposed that a few indefinitely reproducing germ cells at each division produced one germ cell like themselves and one cell with a limited potential, allowing one germ cell to generate many sperm over an extended time (Harris 1929; Tihen 1946). Similar genetic analysis of brood patterns using a sex-linked mutable trait extended the observation, notably, that cluster size was larger when the mutation occurred in a GSC rather than at a later spermatogenic stage (Hartl and Green 1970). The results from this study accurately predicted the existence of 7–10 GSCs per adult testis and indicated that the number of germ-line stem cells stays relatively constant regardless of the number of sperm produced (consistent with earlier predictions, as reviewed in Hannah-Alava 1965). The distribution of brood clusters also suggested that the GSCs divide asynchronously, with quiescent periods of 24–48 hours.

Lineage-tracing experiments using the FLP/FRT site-specific recombination system conclusively verified both the function and location of GSCs in wild-type testes (Fig. 5g) (Gönczy and DiNardo 1996). The FLP recombinase of yeast can be used to induce site-specific recombination at *cis*-acting FRT sites incorporated into *Drosophila* chromosomes (Harrison and Perrimon 1993). The FLP/FRT system can be used to permanently mark cells by inducing mitotic recombination between differently marked homologous chromosomes or by inducing excision of a marker gene from within a single chromosome. Both germ-line stem cells and spermatogonia can be marked in this system. However, spermatogonia are present only transiently, as they eventually clear from the proliferation center within 2 days and differentiate. In contrast, stem cells persist by self-renewal of stem cell identity, continually producing waves of marked progeny undergoing spermatogenesis (Fig. 5g). Such clonal marking studies produced single, persistent, marked germ cells adjacent to the hub at the apical tip in ~50% of the induced testes examined, confirming the identity and location of germ-line stem cells deduced from ultrastructural studies (Gönczy and DiNardo 1996). The function and location of the somatic cyst progenitor stem cells were demonstrated by the same method (Fig. 5h).

The FLP/FRT lineage-tracing system can also be used in mutant genetic backgrounds to identify and follow stem cell behavior in males carrying mutations that cause stem cell defects. In addition, to test whether a gene required for stem cell function acts cell-autonomously or non-cell-autonomously, the same technology can be used to make mosaic animals in which the induced clones are homozygous for a mutation in an otherwise heterozygous background.

Mammals

In mammals, several phenotypic markers are used to distinguish the A_s spermatogonial cells from later spermatogonial stages. GSCs undergoing asynchronous mitosis or apoptosis can be detected as single cells interspersed along the periphery of the seminiferous tubule. In contrast, clusters of interconnected spermatogonia undergo mitosis and enter apoptosis synchronously within each epithelial stage (Huckins 1971b; Oakberg 1971; de Rooij and Grootegoed 1998). Due to the characteristic wavelike distribution of spermatogenic stages along the length of the seminiferous tubule, certain regions show a higher frequency of A_s stem cell proliferation, whereas other epithelial stages contain relatively inactive A_s spermatogonia. The number of A_s spermatogonia, however, remains constant throughout the epithelial cycle (Huckins 1971a). In rat and hamster, male GSCs and undifferentiated spermatogonia generally have a longer cell cycle time than do differentiating spermatogonia (Huckins 1971a; Lok et al. 1983). In rat testes, A_s stem cells are estimated to divide every 60 hours, the A_{pr} and A_{al} undifferentiated spermatogonia every 55 hours, and the differentiating spermatogonia every 42 hours (Huckins 1971a).

A-type spermatogonia, including spermatogonial stem cells, exhibit higher levels of telomerase activity (Ravindranath et al. 1997) and expression of the EE2 protein (Koshimizu et al. 1995) than later germ cell stages. Undifferentiated A spermatogonia can be further distinguished from differentiating spermatogonia on the basis of low nuclear heterochromatin (Huckins 1971b) and low levels of c-Kit expression (Schrans-Stassen et al. 1999).

Transplantation as a Test of Mammalian Germ-line Stem Cell Function

Mammalian germ-line stem cells are able to maintain stem cell function upon transplantation to a host testis. Methods for germ cell transplants have been established in the mouse testis (Brinster and Avarbock 1994;

Brinster and Zimmermann 1994). Although a mixed population of testicular cells is used for the transplant injections, only the spermatogonial stem cells are thought to establish donor-derived spermatogenesis in the host. Donor spermatogonial stem cells can provide long-term reconstitution to produce functional sperm and restore fertility in a sterile host testis, with colonization rates of >80% repopulated tubules in at least 71% of the recipient mice (Brinster and Avarbock 1994). The best colonization occurred when donor cells were injected into hosts depleted of germ cells, obtained either by cytotoxic treatment with busulfan or by genetic mutation with alleles of the *Dominant white spotting* (*W*) or *Steel* (*Sl*) loci (Brinster and Zimmermann 1994; Ohta et al. 2000; Shinohara et al. 2000). These results suggest competition between transplanted and endogenous germ-line stem cells for access to limited sites capable of supporting stem cell function, in support of the niche hypothesis.

The frequency of colonization upon transplantation indicates that germ-line stem cells are rare within the testis, consistent with the estimated two GSCs per every 10^4 germ cells (Meistrich and van Beek 1993a; Tegelenbosch and de Rooij 1993). This frequency compares with the measured frequency of approximately 1 hematopoietic stem cell in every 10^4 bone marrow cells (Spangrude et al. 1988). Individual colonization events upon transplantation can be distinct, allowing the activity of a single stem cell to be followed (Nagano et al. 1999). The ability to visualize individual stem cells verified stem cell localization at the periphery of the tubule lumen (Nagano et al. 1999). Newly transplanted spermatogonia were shown to migrate to the basement membrane, where Sertoli cells extended processes to surround the germ cells (Parreira et al. 1998, 1999).

The transplantation assay has been used to identify purification methods that enrich for male GSCs within populations of spermatogonia. Positive selection for germ cells with high expression of the specific β-1 and α-6 integrins provided a 3- to 5-fold enrichment in transplantable GSCs from mouse testes (Shinohara et al. 1999). β-1 integrin expression also cosegregates with both epidermal and hematopoietic stem cell populations (Jones and Watt 1993; Potocnik et al. 2000). Similarly, selection for binding to laminin also enriched for germ cells capable of stem cell transplantation (Shinohara et al. 1999). A negative selection method utilizing cryptorchid mice, which have a high concentration of undifferentiated spermatogonia, as the transplant donors resulted in a dramatic 50-fold enrichment of transplantable stem cells over controls (Shinohara et al. 2000). Cryptorchidism induced by retention of the testis in the body cavity leads to reversible depletion of differentiating germ cells, apparently without detriment to the stem cell population. The ability to transplant

mammalian germ-line stem cells coupled with the ability to persistently transfect GSCs with retroviral constructs raises the possibility of introducing genetic modifications into the male germ line for either animal transgenics or human gene therapy (Nagano et al. 2000).

MAINTAINING A BALANCE BETWEEN TWO STEM CELL FATES: ASYMMETRIC VERSUS SYMMETRIC DIVISION

Stem cell function is twofold: Stem cells must both maintain the stem cell population and give rise to differentiating cells. The mechanisms that maintain the balance between these two daughter cell fates are the crucial question in stem cell biology. Two prevailing models have been advanced to explain how stem cells give rise to progeny with two different cell fates (Watt and Hogan 2000). The first model proposes that stem cell divisions are asymmetric, ensuring that both cell populations are evenly maintained at every division. An asymmetric outcome upon stem cell division results in one daughter cell that self-renews stem cell identity and another cell that commits to differentiation. This model was first advanced nearly 100 years ago on the basis of cytomorphological evidence in grasshopper spermatogenesis (Davis 1908), then developed by further studies in the fruit fly (Harris 1929). The second model suggests that stem cells normally undergo symmetric divisions (Wilson 1925; Huckins 1971b). Symmetric stem cell divisions must alternate between proliferative divisions, producing two stem cells, and differentiating divisions, resulting in two cells that initiate differentiation (Fig. 2). For this discussion, these models focus on the *outcome* of stem cell division and do not specify whether the mechanisms for stem cell fate decisions are intrinsically determined or extrinsically controlled.

At present, the consensus on male germ-line stem cell division pattern is split between the fly and mammalian systems (de Rooij and Grootegoed 1998; Lin 1998). However, there is not enough evidence to rule out the possibility in either system that male germ-line stem cells normally undergo both asymmetric and/or symmetric divisions.

Fly

Male germ-line stem cells in *Drosophila* usually divide with an asymmetric outcome. As described above, asymmetric stem cell divisions were first proposed to explain the appearance of large, clonal populations obtained from male brood experiments (Harris 1929; Tihen 1946). More recently, the asymmetric outcome of GSC divisions was confirmed by lineage trac-

ing: Single, marked germ cells persisted at the testis apical tip and continually gave rise to differentiated progeny (Fig. 5g,h) (Gönczy and DiNardo 1996). By this criterion, the somatic stem cells also undergo asymmetric divisions. Although it has not yet been analyzed in the male, symmetric proliferative divisions may be possible under conditions that induce stem cell expansion to replace lost stem cells, as was described in the *Drosophila* female germ line (Xie and Spradling 1998, 2000 and Chapter 7).

Both the germ-line stem cells and cyst progenitor cells divide radially within an inherently asymmetric microenvironment (Fig. 3b,c) (Smith and Dougherty 1976; Hardy et al. 1979; Lindsley and Tokuyasu 1980). The cell fates adopted by the two stem cell daughters correlate with their physical location with respect to the hub. Upon germ-line stem cell division, the daughter cell directly adjacent to the hub and enclosed by the cyst progenitor cells retains stem cell identity and self-renewal capacity. The daughter cell displaced away from the hub becomes enclosed in cyst cells and initiates differentiation. A similar spatial relationship between the germ cells and somatic apical cells has been described for the germinal proliferation center in other insects (for review, see Hannah-Alava 1965). The conserved organization and the correlation between physical position and fate suggest that extrinsic cues from surrounding support cells may be important for the asymmetric cell fate decisions made by the two daughters of each stem cell division (see below).

Mammals

It is not yet clear whether mammalian stem cell divisions are normally asymmetric. Historically, three different models have been advanced to explain how mammalian male germ line stem cells maintain steady state (for review, see Meistrich and van Beek 1993a). All models agree that the germ-line stem cells are rare, only 0.03% of the total germ cells. In addition, all models assume that stem cells maintain a steady state so that, despite continual cell division, stem cell numbers do not increase. The A_s model is currently the most widely accepted. According to this model, A_s spermatogonial stem cells usually undergo symmetric divisions (Wilson 1925; Huckins 1971b), resulting in either two self-renewing A_s spermatogonia or two interconnected A_{pr} spermatogonia that initiate differentiation. It is possible that the cell which gives rise to A_{pr} spermatogonia is analogous to the gonialblast in *Drosophila* rather than a true stem cell. The observed delays in spermatogenesis both after irradiation (Meistrich et al. 1978) and after transplantation (Parreira et al. 1998, 1999) indicate

that stem cell expansion precedes initiation of differentiation, suggesting that male GSCs can divide symmetrically to produce two stem cells under these conditions.

MOLECULAR MECHANISMS OF STEM CELL REGULATION: ROLES FOR INTRINSIC VERSUS EXTRINSIC CONTROL

Mutational analysis can be used to identify genes that mediate normal stem cell behavior. Genes required for stem cell specification can be identified in screens for mutations that disrupt stem cell formation. Genes required for the cell fate decisions that specify stem cell self-renewal can be identified in screens for mutations that lead to premature stem cell loss (Fig. 6b). Likewise, genes required for the initiation of differentiation instead of stem cell self-renewal can be identified in screens for mutations that lead to unrestricted proliferation of stem cells (Fig. 6b).

According to the stem cell niche hypothesis, the microenvironment plays a crucial role in regulating stem cell behavior. If so, then genes that are expressed in surrounding somatic support cells that extrinsically regulate germ-line stem cell behavior and genes that act intrinsically within the GSCs are both likely to be required for normal stem cell function. A somatic role in germ-line maintenance was elegantly demonstrated by ablation experiments in the nematode (Kimble and White 1981). Subsequent genetic analysis revealed the underlying molecular mechanism (Henderson et al. 1994). Maintenance of gametogenesis in the nematode syncytial gonad requires expression of the Delta-related ligand *lag-2* in the somatic distal tip cell, which signals via the Notch-like receptor *glp-1* expressed in the germ line to maintain mitotic proliferation of germ cell nuclei (Austin and Kimble 1987). Without the signal from the distal tip cell, the germ cell nuclei cease proliferation and enter meiosis and terminal differentiation.

In both flies and mammals, somatic gonadal cells have been shown to provide important signals for post-stem-cell stages of spermatogenesis. Recent evidence demonstrates that signals from somatic cells also regulate the choice of GSC fates. The best current examples from each system suggest that counteracting signals are likely to balance the choice between stem cell self-renewal and differentiation.

Fly

In *Drosophila*, specification of somatic gonadal precursor cells under control of a genetic hierarchy of pattern formation genes is necessary for

gonad coalescence and subsequent PGC development into germ-line stem cells (Boyle and DiNardo 1995; Boyle et al. 1997). Screens for zygotic genes required for germ cell migration and gonad formation have also identified additional genes that act in the soma (Moore et al. 1998). Although the somatic gonad can form in the absence of germ-line cells (Aboim 1945), the behavior of somatic cells is altered in agametic testes (Gönczy and DiNardo 1996). In testis lacking germ line, cyst cells continue to proliferate and can take on an altered identity. This suggests that cross-regulation between germ cells and somatic cells may coordinate normal germinal proliferation center function. Little is known about the mechanisms that regulate the transition from primordial germ cell to stem cell identity once the germ line is established in the gonad.

A small number of genes specifically required for function of *Drosophila* male germ-line stem cells have been identified from screens for viable, male-sterile mutants. However, since spermatogenic stem cell activity initiates at the end of embryogenesis, genes essential for embryonic viability cannot readily be assessed for effects on male GSC regulation if loss-of-function mutations are homozygous lethal. Chemical mutagenesis can produce specific alleles that affect only the male germ line, weak alleles, or conditional alleles (such as temperature-sensitive alleles) of essential genes to address their roles in spermatogonial stem cell regulation. In addition, production of homozygous mutant clones in otherwise heterozygous males by mitotic recombination (described above) can be used to make gonadal mosaics to test the role of essential genes. Furthermore, the ability to make marked mutant clones in the testis can be used to test whether genes required for GSC regulation act cell-autonomously in the germ line or non-cell-autonomously in surrounding somatic support cells.

Mutations in genes required for survival or maintenance of GSCs cause loss of germ-line renewal. This phenotype can be easily identified by phase microscopy of whole testes. Typically, mutations in which germline stem cells are initially active but are not maintained or become quiescent lead to testes with some differentiating spermatids but loss of the developmental gradient of early germ cells at the apical tip (Fig. 7a,b). Phenotypes can range from few to many sperm, depending on whether a mutation affects establishment of stem cell identity or continual stem cell self-renewal upon division. Additional phenotypic analysis using molecular markers (as in Fig. 5) can then distinguish whether loss of early germ cell differentiation is due to loss of germ-line stem cells, stem cell quiescence, or cell death. Mutants representing all of these classes have been isolated in mutagenesis screens and are currently being characterized.

Mutations in the *Drosophila* gene *piwi* cause loss of renewing germ line in both male and female flies (Lin and Spradling 1997). Males homozygous for *piwi* mutations exhibit tiny testes with a nearly complete absence of spermatogenesis. Piwi protein is normally expressed in the early male germ cells as well as somatic hub and cyst cells (Cox et al.

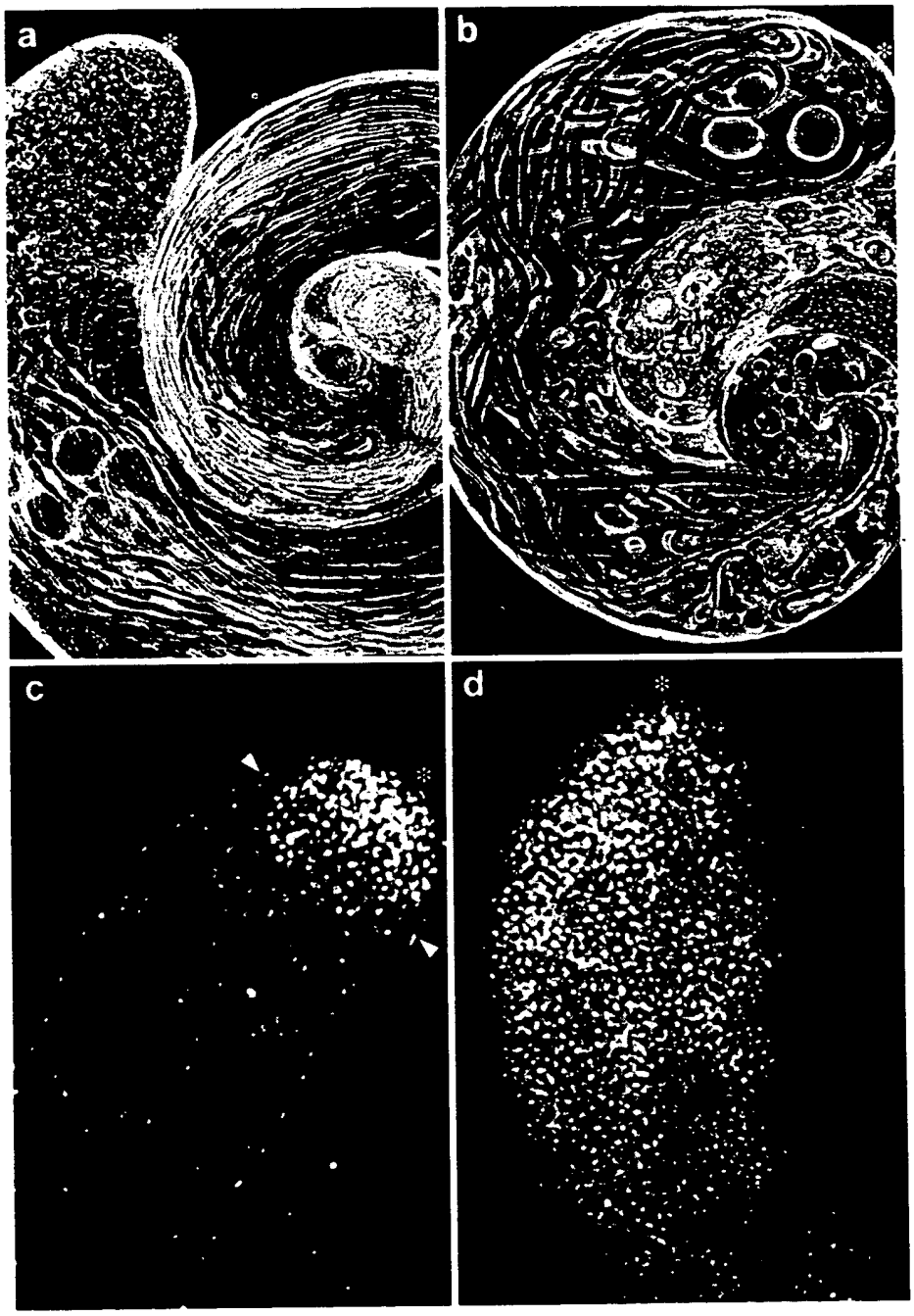

Figure 6 (See facing page for legend.)

2000). Although the male phenotype has not been examined in detail, *piwi* has been shown to be required for both the maintenance and division kinetics of *Drosophila* female GSCs. Wild-type *piwi* function appears to be required both in somatic cells and in the germ line for normal female germ-line stem cell behavior (Cox et al. 1998, 2000). Proteins homologous to Piwi have been found in a wide range of organisms from plants to humans (Cox et al. 1998). The plant homologs, *zwille* and *argonaute*, both play similar roles in maintenance of the plant meristem (Bohmert et al. 1998; Moussian et al. 1998). Mutations in the *Drosophila* gene *escargot* also disrupt maintenance of spermatogenesis; however, a substantial number of sperm are produced before male germ-line stem cells are lost (G. Hime and M. Fuller, unpubl.). Expression of *escargot* mRNA in the testis is restricted to early germ cells and the somatic hub, consistent with either an intrinsic or extrinsic role in male germ-line stem cell maintenance.

An opposite phenotype results from mutation of genes required to specify the gonialblast fate instead of germ-line stem cell self-renewal or to specify further steps of differentiation instead of spermatogonial proliferation. Unrestricted GSC self-renewal or spermatogonial mitotic amplification at the expense of differentiation can be detected by staining for DNA, which reveals expansion of compact, brightly staining mitotic cells at the apical tip (Fig. 6c,d). Overproliferation of stem cells or gonialblasts can be easily distinguished from overproliferation of interconnected spermatogonia using the gene expression, subcellular structure, and cell division behavior markers described above (Fig. 5).

Figure 6 Mutational analysis of *Drosophila* male germ-line stem cell behavior. (*a*) Phase microscopy image of a testis from a wild-type adult containing all stages of spermatogenesis, evidence of continual regeneration due to stem cell activity. Apical tip (*asterisk*). (*b*) Phase microscopy image of a testis from an adult homozygous for a mutation in a gene required for normal stem cell self-renewal, resulting in differentiation and eventual loss of the GSCs. The gradient of round early germ cells including the stem cells is no longer present at the apical tip (*asterisk*), with only sperm bundles remaining. (*c*) Testis from a wild-type adult stained with a fluorescent DNA dye. Testis shows the normal gradient of more compact, brightly staining mitotic germ cells at the apical tip (*asterisk* to *arrowheads*) followed by the more diffusely staining differentiating germ cells. (*d*) Testis from an adult mutant for *Egfr* function and stained for DNA. The testis is filled with mitotic germ cells at the expense of differentiation, seen as the expansion of brightly staining cells throughout the testis.

Genetic analysis has identified several *Drosophila* genes (*bam, bgcn, punt, schnurri*) that control the decision to exit the spermatogonial divisions at the 16-cell stage (Gönczy et al. 1997; Matunis et al. 1997). Function of two genes (*punt* and *schnurri*) is required in the somatic cyst cells to restrict spermatogonial proliferation, indicating extrinsic regulation of germ cell amplification divisions (Matunis et al. 1997). Similar extrinsic mechanisms may limit the proliferative capacity of amplifying progenitors in other lineages, such as the amplifying keratinocytes in the skin or the myeloid progenitors in the bone marrow (Fuchs and Segre 2000; Weissman 2000; Chapter 19).

Recent evidence suggests that a signal(s) from somatic cyst cells also restricts GSC self-renewal and allows differentiation of the gonialblast, thus ensuring that male germ-line stem cell divisions have an asymmetric outcome. Mutations in two different genes suggest that wild-type function of the epidermal growth factor receptor pathway is required in somatic cyst cells for the normal choice of GSC fates (Kiger et al. 2000; Tran et al. 2000). Conditional loss of function of either the *Egfr* or *raf* genes results in a massive increase in the number of early germ cells, including the GSCs and spermatogonia (Fig. 6c,d). In both mutants, many of the accumulated GSCs maintain expression of GSC markers outside of their normal niche alongside the hub or cyst progenitor cells. Accumulation of GSCs and spermatogonia is associated with a block in their differentiation, as no new spermatocytes are observed. Germ-line clones of either *Egfr* or *raf* null alleles demonstrated that wild-type function of these genes was not required in the germ line itself to allow germ-line differentiation. Cyst cell clones homozygous for mutant *raf* resulted in unrestricted proliferation of the encysted germ cells, which still contained a wild-type copy of the *raf* gene (Tran et al. 2000). These results suggest that cyst cells play a guardian role to ensure that upon GSC division, one daughter cell down-regulates the ability to self-renew stem cell identity and adopts a gonialblast fate.

Mammals

In mammals, mutations that cause germ cell depletion or early arrest of spermatogenesis are characterized initially by a "Sertoli-cell only" phenotype—atrophic tubules with few germ cells. The absence of renewing germ line can indicate a failure in germ cell specification, migration, gonad formation, or maintenance of the stem cells. Several genes are known to act extrinsically for primordial germ cell survival; for example, the growth factors LIF and OncM (Hara et al. 1998). The *Dominant white*

spotting (W) and Steel (Sl) receptor–ligand pair are both needed for proper primordial germ cell proliferation and migration. Mutations in either gene result in reduced numbers of primordial germ cells in the embryonic gonad (Russell 1979; Yoshinaga et al. 1991). Interestingly, the *W* and *Sl* mutations also affect the proliferation and migration of erythropoietic and melanocytic precursors (Russell 1979). Chimera experiments testing where c-Kit and Sl function for normal germ cell development demonstrated that the receptor is only required to act in germ cells, whereas the ligand functions in the soma (Nakayama et al. 1988). Although their molecular identity or mechanism of action is not yet known, the Hertwig's anemia (*an*) and atrichosis (*at*) genes are also required for primordial germ cell survival and gonocyte development. Mice mutant for either the *an* or *at* genes have few germ cells due to germ cell death in the embryonic gonad (Chubb and Desjardins 1984; Russell et al. 1985). Finally, disruption of the growth factor BMP8b gene, which is normally expressed in early germ cells, results in a reduction in the number of spermatogonial stem cells established in the embryonic gonad, causing a delay in initiation of spermatogenesis (Zhao et al. 1996).

In other examples, loss of renewing germ line follows relatively normal germ cell specification, gonad formation, and occasionally an initial wave of spermatogenesis. However, the presently known mouse mutations that cause depletion of early germ cells all appear to affect a similar stage just downstream of the stem cells, and not stem cell self-renewal directly (de Rooij et al. 1999). Five distinct situations appear to cause developmental arrest at the switch from undifferentiated A-type spermatogonia (A_{al}) into differentiating-type spermatogonia, resulting in proliferation but not accumulation of undifferentiated A-type spermatogonia in mouse or rat testes (de Rooij et al. 1999; Meistrich et al. 2000). These situations include (1) mutations in the mouse genes *Steel* or *W* (Sl^{17H}, Sl^{d}, and W^{v} alleles) (Koshimizu et al. 1992; de Rooij et al. 1999; Ohta et al. 2000); (2) mutation of the mouse gene *juvenile spermatogonial depletion (jsd)* (Beamer et al. 1988; Mizunuma et al. 1992; de Rooij et al. 1999); (3) conditions of vitamin A deficiency (Huang and Hembree 1979; van Pelt et al. 1995; de Rooij et al. 1999); (4) cryptorchidism (Nishimune and Haneji 1981; de Rooij et al. 1999); and (5) inhibition by intratesticular testosterone after radiation or other toxicant exposures (Meistrich et al. 2000). Colonization of recipient testes with donor GSCs transplanted from *Sl* mutant or cryptorchid mice demonstrated that functional spermatogonial stem cells are in fact retained in these conditions where later stages of differentiating germ cells are depleted (Ohta et al. 2000; Shinohara et al. 2000).

Somatic cells in the testis play an important regulatory role in spermatogenesis, although most hormones and cytokines tested exhibit either pre- or post-spermatogonial effects (Desjardins and Ewing 1993). Less is known about the possible role of somatic cells in regulating spermatogonial stem cell behavior. Recently, the level of glial-cell-line-derived neurotrophic factor (GDNF) produced in Sertoli cells was implicated in regulation of spermatogonial stem cell fate decisions (Meng et al. 2000). Reduction in GDNF function disrupted stem cell maintenance, causing stem cell loss due to differentiation. Conversely, overexpression of GDNF from a transgene construct blocked early germ cell differentiation, resulting in unrestricted proliferation of stem cells and spermatogonia.

Transplantation experiments can be used in mammals to address whether genes that regulate germ-line stem cell behavior are required in the germ line or the somatic lineages. Transplantation of male germ cells between sterile W/W^v and Sl/Sl^d mutant mice demonstrated W is required in germ cells and Sl is required in the soma (Ogawa et al. 2000), in agreement with previous evidence from chimeric mice. Sl/Sl^d mutant germ cells lacking functional ligand were successfully transplanted into sterile W/W^v mutant recipient testes lacking functional receptor (Fig. 7c,d), giving rise to Sl/Sl^d-derived fertile sperm.

The cyclic nature of mammalian spermatogenesis in a given region of seminiferous tubules suggests that reentry into or exit from GSC proliferation must be regulated at distinct stages of the epithelial cycle. An inhibitory activity in testicular extracts capable of blocking the stem cell proliferation that normally reinitiates during mouse epithelial stages II and III has been reported (Clermont and Mauger 1974), possibly derived from the differentiating spermatogonia. Interestingly, the inhibitory factor was tissue- but not species-specific, suggesting that a conserved, testis-specific factor may regulate the timing of GSC divisions. More dramatic regulation of spermatogonial proliferation is seen in certain other seasonally breeding vertebrates, such as the shark (Callard et al. 1989).

Aberrations leading to germ cell neoplasia reveal important points of normal germ-line regulation in humans. Gonadoblastomas occur when PGCs fail to populate the embryonic gonad, such as in XY females, demonstrating the importance of somatic–germ cell interactions in restricting proliferation of GSC precursors (for review, see Pringle and Page 1997). Adult male germ cell tumors may derive from latent gonocytes that transformed into carcinomas in situ (CIS) (de Rooij 1998), suggesting that the developmental transition from a gonocyte to an established male GSC is a critical regulatory step.

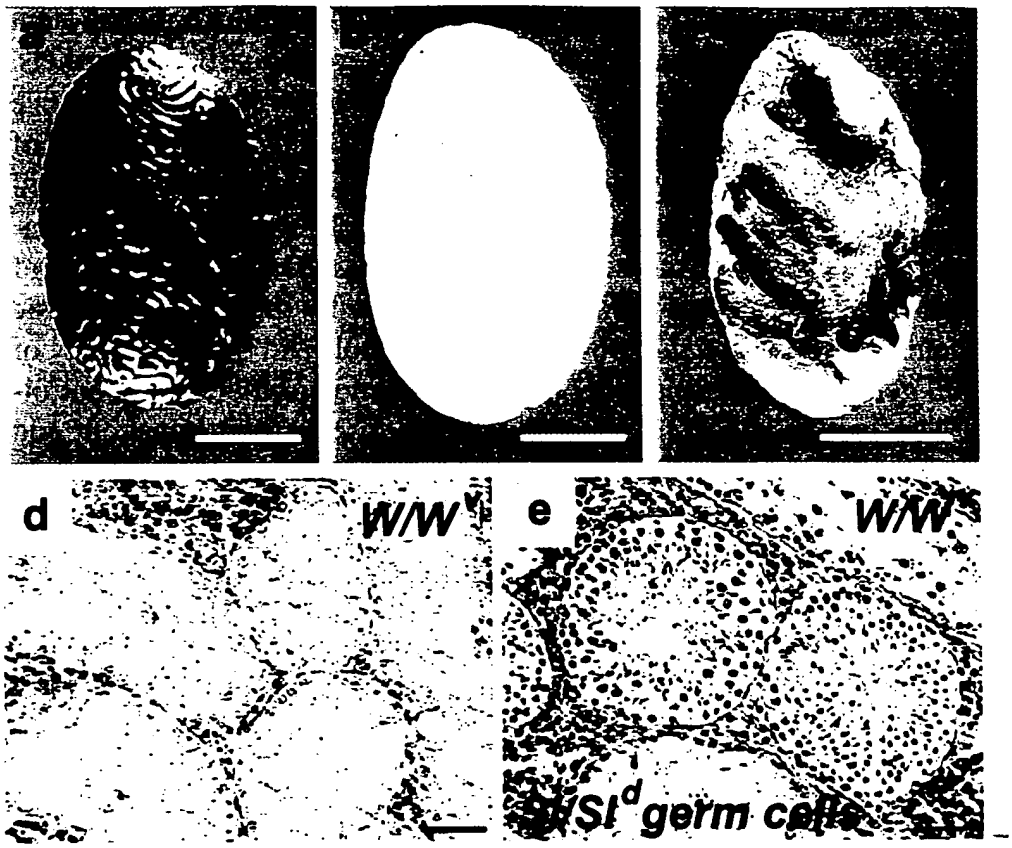

Figure 7 Transplantation of mouse male germ-line stem cells. (*a*) Whole testis from adult ROSA26 transgenic mouse with ubiquitous *lacZ* activity (*blue*) used as donor cells. (*b*) Whole testis from adult mouse without the *lacZ* transgene used as recipient host, demonstrating host cells do not stain. (*c*) Recipient testis 2 months after colonization of transplanted GSCs expressing *lacZ*. Donor-derived colonies of spermatogenesis appear as blue regions within the otherwise white seminiferous tubules of the host. (*d*) Cross-section of a tubule from an adult, sterile *W/W*^{v}mouse testis used as recipient host. Tubules lack spermatogenesis and contain mostly Sertoli cells. (*e*) Spermatogenesis is now evident in most tubules from an adult *W/W*^{v}mouse testis 1 year after transplantation of GSCs from a sterile *Sl/Sl*^{d} donor mouse. The *Sl/Sl*^{d} germ cells, although originally unable to undergo spermatogenesis in the *Sl/Sl*^{d} donor strain, are able to produce fertile sperm in the *W/W*^{v}somatic host environment. (*a*, *b*, *c*, Reprinted, with permission, from Shinohara et al. 1999 [© National Academy of Sciences]; *d*, *e* modified, with permission, from Ogawa et al. 2000.)

SUMMARY AND OUTLOOK

The male germ line offers a powerful system in which to study central questions in stem cell biology. Male germ-line stem cells have been identified in situ, allowing analysis of the role of surrounding somatic support

cells in regulation of stem cell behavior. Analysis of male germ-line stem cell behavior in *Drosophila* and mammals has revealed striking parallels. The ability to combine genetic screens with well-developed descriptive analysis in both these organisms promises functional identification of genes and regulatory pathways that regulate critical aspects of stem cell biology. The ability to construct mosaic animals by germ cell transplantation in mammals or mosaic analysis in *Drosophila* allows tests of whether crucial genes control stem cell behavior by intrinsic or extrinsic mechanisms. In *Drosophila*, powerful genetic tools and the availability of the full genome sequence allow rapid identification of molecules controlling stem cell behavior. In mice, the ability to construct knockout and conditional knockout mutations allows tests of the role of candidate genes in stem cell function. These tools, and the possibility that genes identified by forward genetics in *Drosophila* may have functional homologs that can be tested in mammals, promise to reveal fundamental principles and underlying molecular pathways that may govern stem cell specification, self-renewal, and differentiation in a variety of stem cell systems.

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REFERENCES

Aboim A.N. 1945. Dévelopment embryonnaire et postembryonnaire des gonades normales et agamétique de *Drosophila melanogaster. Rev. Suisse Zool.* **52:** 53–154.

Austin J. and Kimble J. 1987. glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans. Cell* **51:** 589–599.

Bardin W.C., Gunsalus G.L., and Cheng C.Y. 1993. The cell biology of the Sertoli cell. In *Cell and molecular biology of the testis* (ed. C. Desjardins and L.L. Ewing), pp. 189–219. Oxford University Press, Oxford, United Kingdom.

Beamer W.G., Cunliffe-Beamer T.L., Shultz K.L., Langley S.H., and Roderick T.H. 1988. Juvenile spermatogonial depletion (jsd): A genetic defect of germ cell proliferation of male mice. *Biol. Reprod.* **38:** 899–908.

Bohmert K., Camus I., Bellini C., Bouchez D., Caboche M., and Benning C. 1998. AGO1 defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J.* **17:** 170–180.

Boyle M. and DiNardo S. 1995. Specification, migration and assembly of the somatic cells of the *Drosophila* gonad. *Development* **121:** 1815–1825.

Boyle M., Bonini N., and DiNardo S. 1997. Expression and function of clift in the devel-

opment of somatic gonadal precursors within the *Drosophila* mesoderm. *Development* **124:** 971–982.

Brinster R.L. and Avarbock M.R. 1994. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci.* **91:** 11303–11307.

Brinster R.L. and Zimmermann J.W. 1994. Spermatogenesis following male germ-cell transplantation (comments). *Proc. Natl. Acad. Sci.* **91:** 11298–11302.

Brower D.L., Smith R.J., and Wilcox M. 1981. Differentiation within the gonads of *Drosophila* revealed by immunofluorescence. *J. Embryol. Exp. Morphol.* **63:** 233–242.

Callard G., Mak P., DuBois W., and Cuevas M.E. 1989. Regulation of spermatogenesis: The shark testis model. *J. Exp. Zool. Suppl.* **2:** 23–34.

Chubb C. and Desjardins C. 1984. Testicular function and sexual activity in senescent mice. *Am. J. Physiol.* **247:** E569–573.

Clermont Y. 1972. Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* **52:** 198–236.

Clermont Y. and Mauger A. 1974. Existence of a spermatogonial chalone in the rat testis. *Cell Tissue Kinet.* **7:** 165–172.

Clermont Y. and Perey B. 1957. Quantitative study of the cell population of the seminiferous tubules of immature rats. *Am. J. Anat.* **100:** 241–268.

Clermont Y. and Trott M. 1969. Duration of the cycle of the seminiferous epithelium in the mouse and hamster determined by means of 3H-thymidine and radioautography. *Fertil. Steril.* **20:** 805–817.

Cooper K.W. 1950. Normal spermatogenesis in *Drosophila*. In *Biology of* Drosophila (ed. M. Demerec), pp. 1–61. Wiley, New York.

Cox D.N., Chao A., and Lin H. 2000. *piwi* encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127:** 503–514.

Cox D.N., Chao A., Baker J., Chang L., Qiao D., and Lin H. 1998. A novel class of evolutionarily conserved genes defined by *piwi* are essential for stem cell self-renewal. *Genes Dev.* **12:** 3715–3727.

Davis H.P. 1908. Spermatogenesis in the *Acrididae* and *Locustidae*. *Bull. Mus. Comp. Zool. (Harv. Univ.)* **53:** 59–158.

de Rooij D.G. 1998. Stem cells in the testis. *Int. J. Exp. Pathol.* **79:** 67–80.

de Roojj D.G. and Grootegoed J.A. 1998. Spermatogonial stem cells. *Curr. Opin. Cell. Biol.* **10:** 694–701.

de Rooij D.G., Okabe M., and Nishimune Y. 1999. Arrest of spermatogonial differentiation in jsd/jsd, Sl17H/Sl17H, and cryptorchid mice. *Biol. Reprod.* **61:** 842–847.

Desjardins C. and Ewing L.L. 1993. *Cell and molecular biology of the testis*. Oxford University Press, Oxford, United Kingdom.

Dym M. and Clermont Y. 1970. Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. *Am. J. Anat.* **128:** 265–282.

Dym M. and Fawcett D.W. 1970. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol. Reprod.* **3:** 308–326.

———. 1971. Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biol. Reprod.* **4:** 195–215.

Everett N.B. 1943. Observational and experimental evidences relating to the origin and differentiation of the definitive germ cells in mice. *J. Exp. Zool.* **92:** 49–91.

Ewing L.L. and Keeney D.S. 1993. Leydig cell: Structure and function. In *Cell and molecular biology of the testis* (ed. C. Desjardins and L.L. Ewing), pp. 137–165. Oxford

University Press, Oxford, United Kingdom.

Fouquet J.P. and Dadoune J.P. 1986. Renewal of spermatogonia in the monkey (*Macaca fascicularis*). *Biol. Reprod.* **35:** 199–207.

Fuchs E. and Segre J.A. 2000. Stem cells: A new lease on life. *Cell* **100:** 143–155.

Fuller M.T. 1993. Spermatogenesis. In *The development of* Drosophila melanogaster (ed. M. Bate and A. Martinez Arias), vol. 1, pp. 71–147. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Geigy R. 1931. Action de l'ultraviolet sur le pôle germinal dans l'oeuf de *Drosophila melanogaster*. *Rev. Suisse Zool.* **38:** 187–288.

Gilula N.B., Fawcett D.W., and Aoki A. 1976. The Sertoli cell occluding junctions and gap junctions in mature and developing mammalian testis. *Dev. Biol.* **50:** 142–168.

Ginsburg M., Snow M.H., and McLaren A. 1990. Primordial germ cells in the mouse embryo during gastrulation. *Development* **110:** 521–528.

Gönczy P. 1995. "Towards a molecular genetic analysis of spermatogenesis in *Drosophila*." Ph.D. thesis. The Rockefeller University, New York.

Gönczy P. and DiNardo S. 1996. The germ line regulates somatic cyst cell proliferation and fate during *Drosophila* spermatogenesis. *Development* **122:** 2437–2447.

Gönczy P., Matunis E., and DiNardo S. 1997. *bag-of-marbles* and *benign gonial cell neoplasm* act in the germline to restrict proliferation during *Drosophila* spermatogenesis. *Development* **124:** 4361–4371.

Gonczy P., Viswanathan S., and DiNardo S. 1992. Probing spermatogenesis in *Drosophila* with P-element enhancer detectors. *Development* **114:** 89–98.

Hadley M.A. and Dym M. 1987. Immunocytochemistry of extracellular matrix in the lamina propria of the rat testis: Electron microscopic localization. *Biol. Reprod.* **37:** 1283–1289.

Hannah-Alava A. 1965. The premeiotic stages of spermatogenesis. Adv. Genet. **13:** 157–226.

Hara T., Tamura K., de Miguel M.P., Mukouyama Y., Kim H., Kogo H., Donovan P.J., and Miyajima A. 1998. Distinct roles of oncostatin M and leukemia inhibitory factor in the development of primordial germ cells and sertoli cells in mice. *Dev. Biol.* **201:** 144–153.

Hardy R.W., Tokuyasu K.T., Lindsley D.L., and Garavito M. 1979. The germinal proliferation center in the testis of *Drosophila melanogaster*. *J. Ultrastruct Res.* **69:** 180–190.

Harris B.B. 1929. The effects of aging of X-rayed males upon mutation frequency in *Drosophila*. *J. Hered.* **20:** 299–302.

Harrison D.A. and Perrimon N. 1993. Simple and efficient generation of marked clones in *Drosophila*. *Curr. Biol.* **3:** 424–433.

Hartl D.L. and Green M.M. 1970. Genetic studies of germinal mosaicism in *Drosophila melanogaster* using the mutable wc gene. *Genetics* **65:** 449–456.

Hecht N.B. 1993. Gene expression during male germ cell development. In *Cell and molecular biology of the testis* (ed. C. Desjardins and L.L. Ewing), pp. 400–432. Oxford University Press, Oxford, United Kingdom.

Henderson S.T., Gao D., Lambie E.J., and Kimble J. 1994. *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. *Development* **120:** 2913–2924.

Hilscher B., Hilscher W., Bulthoff-Ohnolz B., Kramer U., Birke A., Pelzer H., and Gauss G. 1974. Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and presper-

matogenesis. *Cell Tissue Res.* **154:** 443–470.

Hinton B.T. and Turner T.T. 1993. The seminiferous tubular microenvironment. In *Cell and molecular biology of the testis* (ed. C. Desjardins and L.L. Ewing), pp. 238–265. Oxford University Press, Oxford, United Kingdom.

Huang H.F. and Hembree W.C. 1979. Spermatogenic response to vitamin A in vitamin A deficient rats. *Biol. Reprod.* **21:** 891–904.

Huckins C. 1963. Changes in gonocytes at the time of initiation of spermatogenesis in the rat. *Anat. Rec.* **145:** 243.

———. 1971a. The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. *Cell Tissue Kinet.* **4:** 313–334.

———. 1971b. The spermatogonial stem cell population in adult rats. III. Evidence for a long-cycling population. *Cell Tissue Kinet.* **4:** 335–349.

Huckins C. and Oakberg E.F. 1978. Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. II. The irradiated testes. *Anat. Rec.* **192:** 529–542.

Jones P.H. and Watt F.M. 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* **73:** 713–724.

Jones R.C. and Lin M. 1993. Spermatogenesis in birds. *Oxf. Rev. Reprod. Biol.* **15:** 233–264.

Kerkis J. 1933. Development of gonads in hybrids between *Drosophila melanogaster* and *D. simulans*. *J. Exp. Zool.* **66:** 477–509.

Kiger A.A., White-Cooper H., and Fuller M.T. 2000. Somatic support cells restrict germ line stem cell self-renewal and promote differentiation. *Nature* **407:** 750–754.

Kimble J.E. and White J.G. 1981. On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81:** 208–219.

King R.C. 1970. *Ovarian development in* Drosophila melanogaster. Academic Press, New York.

King R.C. and Akai H. 1971. Spermatogenesis in *Bombyx mori*. I. The canal system joining sister spermatocytes. *J. Morphol.* **124:** 143–166.

Koshimizu U., Watanabe D., Tajima Y., and Nishimune Y. 1992. Effects of W (c-kit) gene mutation on gametogenesis in male mice: Agametic tubular segments in Wf/Wf testes. *Development* **114:** 861–867.

Koshimizu U., Nishioka H., Watanabe D., Dohmae K., and Nishimune Y. 1995. Characterization of a novel spermatogenic cell antigen specific for early stages of germ cells in mouse testis. *Mol. Reprod. Dev.* **40:** 221–227.

Lawson K.A., Dunn N.R., Roelen B.A., Zeinstra L.M., Davis A.M., Wright C.V., Korving J.P., and Hogan B.L. 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* **13:** 424–436.

Leblond C.P. and Clermont Y. 1952a. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. N.Y. Acad. Sci.* **55:** 548–573.

———. 1952b. Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the "periodic acid-fuchsin sulfurous acid" technique. *Am. J. Anat.* **90:** 167–215.

Lee T. and Luo L.1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22:** 451–461.

Li C. and Gudas L.J. 1997. Sequences 5′ of the basement membrane laminin beta 1 chain gene (LAMB1) direct the expression of beta-galactosidase during development of the mouse testis and ovary. *Differentiation* **62:** 129–137.

Lin H. 1997. The tao of stem cells in the germline. *Annu. Rev. Genet.* **31:** 455–491.

———. 1998. The self-renewing mechanism of stem cells in the germline. *Curr. Opin. Cell. Biol.* **10:** 687–693.

Lin H. and Spradling A.C. 1997. A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* **124:** 2463–2476.

Lin H., Yue L., and Spradling A.C. 1994. The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120:** 947–956.

Lin M. and Jones R.C. 1992. Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail, *Coturnix coturnix japonica*. *Cell Tissue Res.* **267:** 591–601.

Lindsley D.T. and Tokuyasu K.T. 1980. Spermatogenesis. In *The genetics and biology of Drosophila* (ed. M. Ashburner and T.R.F. Wright), vol. 20, pp. 225–294. Academic Press, London.

Loeffler M. and Potten C.S. 1997. Stem cells and cellular pedigrees—A conceptual introduction. In *Stem cells* (ed. C.S. Potten), pp. 1–27. Academic Press, London.

Lok D., Jansen M.T., and D.G. de Rooij. 1983. Spermatogonial multiplication in the Chinese hamster. II. Cell cycle properties of undifferentiated spermatogonia. *Cell Tissue Kinet.* **16:** 19–29.

Matsui Y., Zsebo K., and Hogan B.L. 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70:** 841–847.

Matunis E., Tran J., Gönczy P., Caldwell K., and DiNardo S. 1997. *punt* and *schnurri* regulate a somatically derived signal that restricts proliferation of committed progenitors in the germline. *Development* **124:** 4383–4391.

McKearin D. and Ohlstein B. 1995. A role for the *Drosophila* Bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121:** 2937–2947.

Meistrich M.L. and van Beek M.E.A.B. 1993. Spermatogonial stem cells. In *Cell and molecular biology of the testis* (ed. C. Desjardins and L.L. Ewing), pp. 266–295. Oxford University Press, Oxford, United Kingdom.

———. 1993b. Spermatogonial stem cells: Assessing their survival and ability to produce differentiated cells. In *Methods in toxicology* (ed. R.E. Chapin and J. Heindel), vol. 3A, pp. 106–123. Academic Press, New York.

Meistrich M.L., Wilson G., Kangasniemi M., and Huhtaniemi I. 2000. Mechanism of protection of rat spermatogenesis by hormonal pretreatment: Stimulation of spermatogonial differentiation after irradiation. *J. Androl.* **21:** 464–469.

Meistrich M.L., Hunter N.R., Suzuki N., Trostle P.K., and Withers H.R. 1978. Gradual regeneration of mouse testicular stem cells after exposure to ionizing radiation. *Radiat. Res.* **74:** 349–362.

Meng X., Lindahl M., Hyvonen M.E., Parvinen M., de Rooij D.G., Hess M.W., Raatikainen-Ahokas A., Sainio K., Rauvala H., Lakso M., Pichel J.G., Westphal H., Saarma M., and Sariola H. 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287:** 1489–1493.

Mintz B. and Russell E.S. 1957. Gene-induced embryological modifications of primordial germ cells in the mouse. *J. Exp. Zool.* **134:** 207–237.

Mizunuma M., Dohmae K., Tajima Y., Koshimizu U., Watanabe D., and Nishimune Y. 1992. Loss of sperm in juvenile spermatogonial depletion (jsd) mutant mice is ascribed

to a defect of intratubular environment to support germ cell differentiation. *J. Cell Physiol.* **150:** 188–193.

Moore L.A., Broihier H.T., Van Doren M., Lunsford L.B., and Lehmann R. 1998. Identification of genes controlling germ cell migration and embryonic gonad formation in *Drosophila*. *Development* **125:** 667–678.

Moussian B., Schoof H., Haecker A., Jurgens G., and Laux T. 1998. Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. *EMBO J.* **17:** 1799–1809.

Nagano M., Avarbock M.R., and Brinster R.L. 1999. Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biol. Reprod.* **60:** 1429–1436.

Nagano M., Shinohara T., Avarbock M.R., and Brinster R.L. 2000. Retrovirus-mediated gene delivery into male germ line stem cells. *FEBS Lett.* **475:** 7–10.

Nakayama H., Kuroda H., Onoue H., Fujita J., Nishimune Y., Matsumoto K., Nagano T., Suzuki F., and Kitamura Y. 1988. Studies of Sl/Sld in equilibrium with +/+ mouse aggregation chimaeras. II. Effect of the steel locus on spermatogenesis. *Development* **102:** 117–126.

Nishimune Y. and Haneji T. 1981. Testicular DNA synthesis in vivo: Comparison between unilaterally cryptorchid testis and contralateral intact testis in mouse. *Arch. Androl.* **6:** 61–65.

Oakberg E.F. 1956. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99:** 391–414.

———. 1971. Spermatogonial stem-cell renewal in the mouse. *Anat. Rec.* **169:** 515–531.

Ogawa T., Dobrinski I., Avarbock M.R, and Brinster R.L. 2000. Transplantation of male germ line stem cells restores fertility in infertile mice. *Nat. Med.* **6:** 29–34.

Ohta H., Yomogida K., Dohmae K., and Nishimune Y. 2000. Regulation of proliferation and differentiation in spermatogonial stem cells: The role of c-kit and its ligand SCF. *Development* **127:** 2125–2131.

Orth J.M. 1982. Proliferation of Sertoli cells in fetal and postnatal rats: A quantitive autoradiographic study. *Anat. Rec.* **203:** 485–492.

Orth J.M. 1993. Cell biology of testicular development in the fetus and neonate. In *Cell and molecular biology of the testis* (ed. C. Desjardins and L.L. Ewing), pp. 3–57. Oxford University Press, Oxford, United Kingdom.

Parreira G.G., Ogawa T., Avarbock M.R., Franca L.R., Brinster R.L., and Russell L.D. 1998. Development of germ cell transplants in mice. *Biol. Reprod.* **59:** 1360–1370.

Parreira G.G., Ogawa T., Avarbock M.R., Franca L.R., Hausler C.L., Brinster R.L., and Russell L.D. 1999. Development of germ cell transplants: Morphometric and ultrastructural studies. *Tissue Cell* **31:** 242–254.

Phillips D.M. 1970. Insect sperm: Their structure and morphogenesis. *J. Cell. Biol.* **44:** 243–277.

Poirie M., Niederer E., and Steinmann-Zwicky M. 1995. A sex-specific number of germ cells in embryonic gonads of *Drosophila*. *Development* **121:** 1867–1873.

Potocnik A.J., Brakebusch C., and Fässler R. 2000. Fetal and adult hematopoietic stem cells require 1 integrin function for colonizing fetal liver, spleen, and bone marrow. *Immunity* **12:** 653–663.

Pringle M.J. and Page D.C. 1997. Somatic and germ cell sex determination in the developing gonad. In *Infertility in the male* (ed. L.I. Lipshultz and S.S. Howards), pp. 3–22.

Mosby, St. Louis, Missouri.

Pudney J. 1995. Spermatogenesis in nonmammalian vertebrates. *Microsc. Res. Tech.* **32:** 459–497.

Ravindranath N., Dalal R., Solomon B., Djakiew D., and Dym M. 1997. Loss of telomerase activity during male germ cell differentiation. *Endocrinology* **138:** 4026–4029.

Resnick J.L., Bixler L.S., Cheng L., and Donovan P.J. 1992. Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359:** 550–551.

Rongo C., Broihier H.T., Moore L., Van Doren M., Forbes A., and Lehmann R. 1997. Germ plasm assembly and germ cell migration in *Drosophila*. *Cold Spring Harbor Symp. Quant. Biol.* **62:** 1–11.

Roussel J.D., Parrott M.W., and Tuttle L.W. 1969. A preliminary study of injury and recovery of the male germinal epithelium and spermatogenesis in *Macaca mulatta* following whole body cobalt-60 gamma irradiation. *J. Reprod. Fertil.* **18:** 177–178.

Russell E.S. 1979. Hereditary anemias of the mouse: A review for genetics. *Adv. Genet.* **20:** 357–459.

Russell E.S., McFarland E.C., and Peters H. 1985. Gametic and pleiotropic defects in mouse fetuses with Hertwig's macrocytic anemia. *Dev. Biol.* **110:** 331–337.

Russell L.D., Tallon-Doran M., Weber J.E., Wong V., and Peterson R.N. 1983. Three-dimensional reconstruction of a rat stage V Sertoli cell: III. A study of specific cellular relationships. *Am. J. Anat.* **167:** 181–192.

Saffman E.E. and Lasko P. 1999. Germline development in vertebrates and invertebrates. *Cell Mol. Life Sci.* **55:** 1141–1163.

Schrans-Stassen B.H., van de Kant H.J., de Rooij D.G., and van Pelt A.M. 1999. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology* **140:** 5894–5900.

Shamblott M.J., Axelman J., Wang S., Bugg E.M., Littlefield J.W., Donovan P.J., Blumenthal P.D., Huggins G.R., and Gearhart J.D. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells (erratum *Proc. Natl. Acad. Sci.* [1999] **96:**1162). *Proc. Natl. Acad. Sci.* **95:** 13726–13731.

Shinohara T., Avarbock M.R., and Brinster R.L. 1999. β_1- and α_6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl. Acad. Sci.* **96:** 5504–5509.

———. 2000. Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. *Dev. Biol.* **220:** 401–411.

Skinner M.K., Tung P.S., and Fritz I.B. 1985. Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. *J. Cell. Biol.* **100:** 1941–1947.

Smith P.A. and Dougherty J.F. 1976. The premeiotic stages of spermatogenesis in *Drosophila melanogaster*. *Amer. Zool.* **16:** 189.

Sonnenblick B.P. 1941. Germ cell movements and sex determination of the gonads in the *Drosophila* embryo. *Proc. Natl. Acad. Sci.* **26:** 373–381.

Spangrude G.J., Heimfeld S., and Weissman I.L. 1988. Purification and characterization of mouse hematopoietic stem cells (erratum *Science* [1989] **244:**1030). *Science* **241:** 58–62.

Staab S., Heller A., and Steinmann-Zwicky M. 1996. Somatic sex-determining signals act on XX germ cells in *Drosophila* embryos. *Development* **122:** 4065–4071.

Stern C. 1941. The growth of the testes in *Drosophila*: I. The relation between vas deferens and testis within various species. *J. Exp. Zool.* **87:** 113–158.

Tam P.P. and Snow M.H. 1981. Proliferation and migration of primordial germ cells dur-

ing compensatory growth in mouse embryos. *J. Embryol. Exp. Morphol.* **64:** 133–147.
Tegelenbosch R.A. and de Rooij D.G. 1993. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat. Res.* **290:** 193–200.
Tihen J.A. 1946. An estimate of the number of cell generations preceding sperm formation in *Drosophila melanogaster*. *Am. Nat.* **80:** 389–393.
Tran J., Brenner T.J., and DiNardo S. 2000. Somatic control over the germline stem cell lineage during *Drosophila* spermatogenesis. *Nature* **407:** 764–757.
Upadhyay S.N. and Guraya S.S. 1973. Histochemical studies on the spermatogenesis of some telecost fishes. *Acta. Anat.* **86:** 484–514.
van Dissel-Emiliani F.M., de Boer-Brouwer M., Spek E.R., van der Donk J.A., and de Rooij D.G. 1993. Survival and proliferation of rat gonocytes in vitro. *Cell Tissue Res.* **273:** 141–147.
van Haaster L.H. and de Rooij D.G. 1994. Partial synchronization of spermatogenesis in the immature Djungarian hamster, but not in the immature Wistar rat. *J. Reprod. Fertil.* **101:** 321–326.
van Pelt A.M., van Dissel-Emiliani F.M., Gaemers I.C., van der Burg M.J., Tanke H.J., and de Rooij D.G. 1995. Characteristics of A spermatogonia and preleptotene spermatocytes in the vitamin A-deficient rat testis. *Biol. Reprod.* **53:** 570–578.
Watt F.M. and Hogan B.L. 2000. Out of Eden: Stem cells and their niches. *Science* **287:** 1427–1430.
Weissman I.L. 2000. Stem cells: Units of development, units of regeneration, and units in evolution. *Cell* **100:** 157–168.
Wilson E.B. 1925. *The cell in development and heredity*. Macmillan, New York.
Wylie C. 1999. Germ cells. *Cell* **96:** 165–174.
Xie T. and Spradling A.C. 1998. decapentaplegic is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell* **94:** 251–260.
Xie T. and Spradling A.C. 2000. A niche maintaining germline stem cells in the *Drosophila* ovary. *Science* **290:** 328–330.
Xu T. and Rubin G.M. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117:** 1223–1237.
Yoshinaga K., Nishikawa S., Ogawa M., Hayashi S., Kunisada T., and Fujimoto T. 1991. Role of *c-kit* in mouse spermatogenesis: Identification of spermatogonia as a specific site of *c-kit* expression and function. *Development* **113:** 689–699.
Zamboni L. and Merchant H. 1973. The fine morphology of mouse primordial germ cells in extragonadal locations. *Am. J. Anat.* **137:** 299–335.
Zhao G.Q., Deng K., Labosky P.A., Liaw L., and Hogan B.L. 1996. The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev.* **10:** 1657–1669.

EXHIBIT D

1: Hum Gene Ther 1997 Feb 10;8(3):285-91

Coupled effects of polybrene and calf serum on the efficiency of retroviral transduction and the stability of retroviral vectors.

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The relative concentrations of Polybrene (PB) and calf serum (CS) in retroviral supernatant have considerable effects on the efficiency of retrovirus-mediated gene transfer and the stability of retroviral vectors. The effect of PB on the efficiency of transduction of Moloney murine leukemia virus (MMuLV)-derived vectors is strongly dependent on CS. At a fixed CS concentration, the efficiency of transduction shows a maximum as a function of PB concentration. Increasing the CS concentration shifted this maximum to higher PB concentrations, but the value of the maximum remained the same. Therefore, there were optimal combinations of PB and CS concentrations that maximized the efficiency of gene transfer: 4.4, 8.8, 13.2, and 22 micrograms/ml of PB for 1%, 2.5%, 5%, and 10% (vol/vol) CS, respectively. Moreover, the presence of PB affected significantly the kinetics of retroviral decay. The loss of retroviral activity did not follow simple exponential decay in the absence of PB during the decay period of the viral supernatant. The dynamics of viral inactivation showed an initial phase during which the transduction efficiency remained constant followed by exponential decay. However, in the presence of high PB concentrations (13.2 micrograms/ml) during the decay period of retroviral vectors, the initial delay was lost and the decay was exponential right from the outset. The present results suggest that in addition to virus-cell interactions that occur on the target cell surface, other physico-chemical processes may occur in solution that have profound effect on retroviral activity and therefore they are of particular importance for gene therapy.

PMID: 9048195 [PubMed - indexed for MEDLINE]

EXHIBIT E

1: Hum Gene Ther 1998 Jan 20;9(2):225-34

High-level gene transfer to cord blood progenitors using gibbon ape leukemia virus pseudotype retroviral vectors and an improved clinically applicable protocol.

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The best methods for transducing hematopoietic progenitor cells usually involve either direct co-cultivation with virus-producing cells or human stromal supportive cells. However, these methods cannot be safely or easily applied to clinical use. Therefore, we aimed at improving retrovirus-mediated gene transfer into hematopoietic progenitors derived from cord blood CD34+ cells using viral supernatant to levels achieved at least with direct co-cultivation and under conditions that are suitable for clinical applications. In a first set of experiments, CD34+ cells were infected with supernatant containing amphotropic retroviral particles carrying the nls-lacZ reporter gene and the effects of centrifugation, cell adhesion to fibronectin, and Polybrene on the transduction of both clonogenic progenitors (CFC) and long-term culture initiating cells (LTC-IC) were studied. Transduction efficiency was evaluated on the percentage and total number of progenitors expressing the beta-galactosidase activity. Results show that a 48-hr infection of CD34+ cells with viral supernatant combining centrifugation at 1000 x g for 3 hr followed by adhesion to fibronectin allows transduction levels for both CFC and LTC-IC to be reached that are as good as using direct co-cultivation. In a second set of experiments, CD34+ cells were infected using this optimized protocol with pseudotyped retroviral particles carrying the gibbon ape leukemia virus (GALV) envelope protein. Under these conditions, between 50 and 100% of CFC and LTC-IC were transduced. Thus, we have developed a protocol capable of highly transducing cord blood progenitors under conditions suitable for a therapeutical use.

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EXHIBIT F

1: Proc Natl Acad Sci U S A 2002 Dec 10;99 Suppl 4:16433-7

The insulation of genes from external enhancers and silencing chromatin.

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Insulators are DNA sequence elements that can serve in some cases as barriers to protect a gene against the encroachment of adjacent inactive condensed chromatin. Some insulators also can act as blocking elements to protect against the activating influence of distal enhancers associated with other genes. Although most of the insulators identified so far derive from Drosophila, they also are found in vertebrates. An insulator at the 5' end of the chicken beta-globin locus marks a boundary between an open chromatin domain and a region of constitutively condensed chromatin. Detailed analysis of this element shows that it possesses both enhancer blocking activity and the ability to screen reporter genes against position effects. Enhancer blocking is associated with binding of the protein CTCF; sites that bind CTCF are found at other critical points in the genome. Protection against position effects involves other properties that appear to be associated with control of histone acetylation and methylation. Insulators thus are complex elements that can help to preserve the independent function of genes embedded in a genome in which they are surrounded by regulatory signals they must ignore.

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EXHIBIT 6

Biochem. Soc. Symp. 67, 195–202
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18

Transgenic mouse models of Alzheimer's disease: phenotype and mechanisms of pathogenesis

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Abstract

A range of transgenic mice have been created to model Alzheimer's disease. These include mice expressing human forms of the amyloid precursor protein, the presenilins and, more recently, tau. Several of the models develop features of the disease including amyloid pathology, cholinergic deficits, neurodegeneration and cognitive impairment. Progress in the characterization and use of these model animals is discussed.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease. Most cases of AD occur sporadically, but familial forms of the disease have been most widely studied because of the insight they give into disease aetiology. Genetic causes of the disease are heterogeneous and include mutations or variants in several genes including those for amyloid precursor protein (APP), the presenilins (PS) and apolipoprotein E [1]. The disease phenotype is remarkably consistent and includes the accumulation of β-amyloid (Aβ) and its deposition into senile plaques, the formation of tau-containing tangles, reactive gliosis, neurodegeneration, cholinergic deficit and cognitive impairment.

The first transgenic mouse to develop a robust AD-related phenotype was described in 1995 by the Exemplar/Athena Neuroscience group [2]. This line known as PDAPP, overexpresses mutant APP at levels high enough to generate sufficient Aβ for extracellular deposits (plaques) to form in relevant regions of the brain. In 1996, a second line (Tg2576) was created by Karen Hsiao and colleagues, which also made sufficient amyloid for deposits to form. In addition, this mouse showed age-related cognitive impairment [3]. Subsequently, other cDNA mice [4,5] and mice overexpressing genomic con-

structs [6] have also been shown to form amyloid in old age. Several groups have created transgenic mice that overexpress mutant PS [7–9] but these mice do not show amyloid deposition, most likely because they have insufficient levels of the Aβ peptide.

Most of the current work on transgenic mice focuses on the cellular response to amyloid accumulation and its relevance to AD. Very recently, the PDAPP line of mice has been used to test the feasibility of modulating amyloid levels by immunization with Aβ [10]. The results of this experiment suggest that amyloid modulation is indeed possible, and that some of the secondary effects of amyloidosis (gliosis and neuritic changes) can be prevented. This work opens up a new direction in amyloid research and may well have a significant impact on the development of human therapies.

Recent advances in phenotype assessment in transgenic models of AD

Amyloidosis

Several studies aimed to modulate the amyloid phenotype by crossing in other transgenes such as PS1 or transforming growth factor-β. The studies showed that when a PS1 mutant mouse was crossed with a mouse overexpressing APP, the levels of Aβ42/43 (the 42/43-amino-acid Aβ peptide) were increased in the double transgenic. This elevation had a profound influence on the age at which amyloid deposition could first be detected [6,11,12]. In one cross, the age at which amyloid deposits were first identified was reduced from 9–12 months to 10–12 weeks [12a], which is the earliest age at which amyloid has been reported. When transforming growth factor-β cDNA mice are crossed with a line overexpressing APP, amyloid deposition was again accelerated and was far more prominent in the vasculature [13]. Apart from demonstrating that these pathways interact, one outcome of the crossed-mouse studies is to enhance, and in some cases modify, the phenotype, providing us with better models.

Presenilin transgenics

In terms of the amyloid phenotype seen in AD, the most significant phenotype in the mutant PS transgenics continues to be the specific elevation of Aβ42/43 [7–9]. Mutant PS2 transgenics that show elevation in Aβ42/43 have also recently been created [14], which strengthens the argument that APP and the presenilins interact, either directly as suggested by Wolfe et al. [15] or indirectly, and that PS mutations cause AD through APP/Aβ modulation.

Studies of knockout PS1 animals have shown that PS1 plays an important role in development. Lack of the protein leads to a deficiency in somitogenesis during early embryogenesis, which results in severe skeletal abnormalities and prenatal death [16,17]. These abnormalities strongly resemble those seen in Notch knockout mice [18] and this observation, coupled with several studies *in vivo* and *in vitro*, suggests that Notch and PS1 interact in some way to affect normal cellular function, which downstream may affect signalling, differentia-

tion and development [19].

PS1 has been strongly implicated in other signalling pathways as several potential components of signal transduction pathways have been identified as PS-interacting proteins. These include several proteins containing an armadillo repeat region, a 42-amino-acid motif that has been identified in proteins involved in cell-to-cell adhesion, protein–protein interaction and signal transduction. The best known of these is β-catenin. Both β-catenin, and its homologue δ-catenin, have been shown to interact with PS1 [20]. The effect of PS1 mutations on β-catenin stability, and hence its downstream effects, are controversial. In transgenic mice and human familial AD brain homogenates, for example, mutations in PS1 are linked to increased degradation of β-catenin [21], whereas other studies have suggested no effect or increased activity for the wild-type and mutant PS1 protein [22–24]. It remains unclear quite how PS1 mutations might lead to AD through a β-catenin pathway, although in addition to the effect on Aβ42 they are thought to affect the action of glycogen synthase kinase-β and hence tau phosphorylation (for a review, see Alzheimer forum panel discussion at www.alzforum.org/members/forums/journals/catenin/index.html).

Neurodegeneration

The human AD brain shows extensive neurodegeneration, both in cholinergic neurons of the nucleus basalis [25], and in non-cholinergic neurons throughout the cortex and hippocampus. Studies have shown that fibrillar Aβ peptides are toxic to neurons in culture [26] and the overproduction of human Aβ in the brains of transgenic mice was therefore expected to cause extensive neurodegeneration. Several of the best characterized mouse models have been examined for overt cell loss [27–29] but neither PDAPP, Tg2576 nor the Tg2576/PS1 cross-mouse show significant cell loss, even though amyloid burden exceeds 30%. One model has been reported to show significant cell loss, but only in the hippocampus [29]. Although overt cell loss is not seen in mice such as Tg2576/PS1, neurites in close proximity to amyloid deposits are severely dystrophic [29a]. In addition, magnetic resonance imaging (MRI) has revealed differences in the volume of structures such as the lateral ventricles and the corpus callosum between mice with and without amyloid, which may reflect loss of neuropil or shrinkage of cells rather than cell death itself (J. Helpern, K. Duff, R. Nixon, T. Wisniewski and M. De Leon, unpublished work). Although the field is in its infancy, the application of functional and structural MRI to the analysis of models is predicted to have a significant impact, especially as longitudinal analyses, including the effects of drug treatments, can be performed on the same mouse.

Cholinergic deficits

Although modulation of the cholinergic system has been a therapeutic treatment for AD dementia for many years, investigations into the response of

cholinergic neurons to amyloid insult have only recently been performed in transgenic mice. Immunohistochemical analysis has shown that cholinergic markers accumulate in swollen abnormal neurites around amyloid deposits in the cortex [4]. Our own studies have shown that in the early stages of deposition, neurons in the nucleus basalis of depositing mice appear normal, but their projection areas in certain regions of the cortex show a significant reduction in synapse density and size [30]. Further work to assess how cholinergic neurons in all areas of the brain respond to increasing amyloid burden and age is underway as cholinesterase inhibitors are currently considered to be valid therapeutic agents for AD.

Cognitive impairments and neurodegeneration

Recreating human cognitive impairment is perhaps the greatest challenge facing genetic engineers working on transgenic models of human dementia. Mice are genetically less suitable for behavioural testing than rats as performance data on normal mice from different strains are often contradictory. Despite these reservations, most of the transgenic mice that form amyloid deposits have been tested for cognitive impairment. The PDAPP mouse [31,32], Tg2576 [3] and the Tg2576/PS1 cross-mouse [12] have all shown a deficit in tests of hippocampal dysfunction before amyloid deposits form, which strongly suggests that overt amyloid accumulation/deposition is not responsible for this early cognitive impairment. Deficits in water maze performance that correlate with increasing age and amyloid burden and with decreasing long-term potentiation have been reported for Tg2576 [3,33]. Recent studies with Tg2576 and the Tg2576/PS1 cross suggest that amyloid/age-related deficits in tests such as the radial arm maze (and especially the radial arm water maze) may be more informative than the regular Morris water maze and that appreciable cognitive impairment may be a feature of mice with extensive amyloid deposits (D. Quartermain, T. Wisneiwski and K. Duff, unpublished work).

Tau pathology

One of the major deficits in the current AD mice is the lack of tau pathology. In humans, tau pathology takes the form of intracellular tangles of an abnormally phosphorylated form of the tau protein, which associates into paired helical filaments. Amyloid plaques and tau tangles are both pathognomic features of human AD, and their relative contribution to the disease has long been disputed. The identification of AD-causing mutations in APP and the presenilins, however, adds weight to the amyloid based hypothesis of pathogenesis, which assumes that tau abnormalities are a secondary lesion that form in response to amyloid accumulation. To investigate this link, transgenic mice with extensive amyloid burden have been examined for abnormal tau pathology by immunohistochemical analysis. This work has shown that amyloid deposits in transgenic mice are ringed by dystrophic neurites that are

immunoreactive for markers of phospho-tau epitopes such as phosphoserine[202] [4,34]. These epitopes are phosphorylated to some degree in the normal brain, but are hyperphosphorylated in the AD brain. In the mice, it is not yet clear whether the immunoreactivity around deposits reflects local hyperphosphorylation of tau, or simple elevation of tau protein levels in response to neuritic damage. In the human AD brain, subsets of neurons are also immunolabelled with antibodies to both tyrosine phospho-tau and the signalling protein fyn which is an src, non-receptor tyrosine kinase [35]. Co-immunoprecipitation has shown that the N-terminus of tau and the SH3 (src homology) domain of fyn interact directly, suggesting that tau may be involved in signal transduction pathways [36]. Interestingly, fyn binds another signalling protein, FAK [37], which is itself up-regulated by Aβ [38]. Ongoing work includes a study of how FAK, fyn, tau and Aβ interact in transgenic animals with and without elevated amyloid.

It is clear, however, that tau pathology does not develop further in the transgenic animals. This suggests that either Aβ/amyloid accumulation is not detrimental to tau or differences between the mouse and human brain preclude the formation of pathogenic tau as suggested by Yankner and colleagues [39]. We have recently created a line of transgenic mice that overexpress all isoforms of human tau under the control of the human tau promoter [40]. These mice not only provide a humanized tau environment in which amyloid deposition can be elicited through crossbreeding, but they also generate animal models in which both normal and abnormal tau biology can be studied. The latter is perhaps particularly significant in light of the recent identification of mutations in tau that cause frontotemporal dementia with Parkinsonism linked to chromosome 17 [41–43], as some of the mutations result in an imbalance of the normal tau isoform ratios; a situation that is reproduced in the transgenic mice.

Summary

Transgenic models of AD continue to gain credibility as more features of the human disease are shown to be represented in the mice. They are, however, still incomplete models as neither tau pathology nor extensive cell loss has been generated in the models created so far. Despite these shortcomings, they are excellent models of amyloidosis and have been highly informative in advancing our understanding of *in vivo* responses to amyloid insult, and the mechanism by which other AD-related genes cause the disease.

References

1. Cruts, M. and Van Broeckhoven, C. (1998) Molecular genetics of Alzheimer's disease. *Ann. Med.* 30, 560–565
2. Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemena, J., Donaldson, T., Gillespie, F., et al. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F β-amyloid precursor protein. *Nature (London)* 373, 523–527
3. Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F. and Cole, G. (1996) Correlative memory deficits, Aβ elevation and amyloid plaques in transgenic mice. *Science* 274, 99–102
4. Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K.H., Mistl, C., Rothacher, S., Ledermann, B., Burki, K., Frey, P., Paganetti, P.A., et al. (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proc. Natl. Acad. Sci. U.S.A.* 94, 13287–13292
5. Nalbantoglu, J., Tirado-Santiago, G., Lahsaini, A., Poirier, J., Goncalves, O., Verge, G., Momoli, F., Welner, S.A., Massicotte, G., Julien, J.P. and Shapiro, M.L. (1997) Impaired learning and LTP in mice expressing the carboxy terminus of the Alzheimer amyloid precursor protein. *Nature (London)* 387, 500–505
6. Lamb, B.A., Bardel, K.A., Kulnane, L.S., Anderson, J.J., Holtz, G., Wagner, S.L., Sisodia, S.S. and Hoeger, E.J. (1999) Amyloid production and deposition in mutant amyloid precursor protein and presenilin-1 yeast artificial chromosome transgenic mice. *Nat. Neurosci.* 8, 695–697
7. Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C.M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., et al. (1996) Increased amyloid-Aβ42(43) in brains of mice expressing mutant presenilin 1. *Nature (London)* 383, 710–713
8. Borchelt, D.R., Thinakaran, G., Eckman, C.B., Lee, M.K., Davenport, F., Ratovitsky, T., Prada, C.M., Kim, G., Seekins, S., Yager, D., et al. (1996) Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo. *Neuron* 17, 1005–1013
9. Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., et al. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. *Nat. Med. (N.Y.)* 3, 67–68
10. Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., et al. (1999) Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature (London)* 400, 173–177
11. Borchelt, D.R., Ratovitski, T., van Lare, J., Lee, M.K., Gonzales, V., Jenkins, N.A., Copeland, N.G., Price, D.L. and Sisodia, S.S. (1997) Accelerated amyloid deposition in the brains of transgenic mice co-expressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 19, 939–945
12. Holcomb, L., Gordon, M.N., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., et al. (1998) Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat. Med. (N.Y.)* 4, 97–100

12a McGowan, E., Sanders, S., Iwatsubo, T., Takeuchi, A., Saido, T., Zehr, C., Yu, X., Uljon, S.E., Wang, R., Mann, D., et al. (1999) Amyloid phenotype characterization of transgenic mice over-expressing both mutant amyloid precursor protein and mutant presenilin 1 transgenes. *Neurobiol. Dis.* 6, 231–244

13. Wyss-Coray, T., Masliah, E., Mallory, M., McConlogue, L., Johnson-Wood, K., Lin, C. and Mucke, L. (1997) Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. *Nature (London)* 389, 603–606

14. Oyama, F., Sawamura, N., Kobayashi, K., Morishima-Kawashima, M., Kuramochi, T., Ito, M., Tomita, T., Maruyama, K., Saido, T.C., Iwatsubo, T., et al. (1998) Mutant presenilin 2 transgenic mouse: effect on an age-dependent increase of amyloid beta-protein 42 in the brain. *J. Neurochem.* 71, 313–322
15. Wolfe, M.S., Xia, W., Ostaszewski, B.L., Diehl, T.S., Kimberly, W.T. and Selkoe, D.J. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature (London)* 398, 513–517
16. Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J., Trumbauer, M.E., Chen, H.Y., Price, D.L., Van der Ploeg, L.H. and Sisodia, S.S. (1997) Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. *Nature (London)* 387, 288–292
17. Shen, J., Bronson, R.T., Chen, D.F., Xia, W., Selkoe, D.J. and Tonegawa, S. (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell* 89, 629–639
18. Conlon, R.A., Reaume, A.G. and Rossant, J. (1995) Notch1 is required for the coordinate segmentation of somites. *Development* 121, 1533–1545
19. Hardy, J. and Israel, A. (1999) In search of gamma secretase. *Nature (London)* 398, 466–467
20. Zhou, J., Liyanage, U., Medina, M., Ho, C., Simmons, A.D., Lovett, M. and Kosik, K.S. (1997) Presenilin 1 interaction in the brain with a novel member of the Armadillo family. *NeuroReport* 8, 2085–2090
21. Zhang, Z., Hartmann, H., Do, V.M., Abramowski, D., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., van de Wetering, M., Clevers, H., Saftig, P., et al. (1998) Destabilization of beta-catenin by mutations in presenilin-1 potentiates neuronal apoptosis. *Nature (London)* 395, 698–702
22. Murayama, M., Tanaka, S., Palacino, J., Murayama, O., Honda, T., Sun, X., Yasutake, K., Nihonmatsu, N., Wolozin, B. and Takashima, A. (1998) Direct association of presenilin-1 with beta-catenin. *FEBS Lett.* 433, 73–77
23. Kang, D.E., Soriano, S., Frosch, M.P., Collins, T., Naruse, S., Sisodia, S.S., Leibowitz, G., Levine, F. and Koo, E.H. (1999) Presenilin 1 facilitates the constitutive turnover of beta-catenin: differential activity of Alzheimer's disease-linked PS1 mutants in the beta-catenin-signaling pathway. *J. Neurosci.* 19, 4229–4237
24. Nishimura, M., Yu, G., Levesque, G., Zhang, D.M., Ruel, L., Chen, F., Milman, P., Holmes, E., Liang, Y., Kawarai, T., et al. (1999) Presenilin mutations associated with Alzheimer disease cause defective intracellular trafficking of beta-catenin, a component of the presenilin protein complex. *Nat. Med. (N.Y.)* 5, 164–169
25. Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T. and DeLong, M.R. (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain, *Science* 215, 1237–1239
26. Lorenzo, A. and Yankner, B.A. (1994) β-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc. Natl. Acad. Sci. U.S.A.* 91, 12243–12247
27. Irizarry, M.C., Soriano, F., McNamara, M., Page, K.J., Schenk, D., Games, D. and Hyman, B.T. (1997) Aβ deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. *J. Neurosci.* 17, 7053–7059
28. Irizarry, M.C., McNamara, M., Fedorchak, K., Hsiao, K. and Hyman, B.T. (1997) APPSw transgenic mice develop age-related Aβ deposits and neuropil abnormalities, but no neuronal loss in CA1. *J. Neuropathol. Exp. Neurol.* 56, 965–973
29. Calhoun, M.E., Wiederhold, K.H., Abramowski, D., Phinney, A.L., Probst, A., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B. and Jucker, M. (1998) Neuron loss in APP transgenic mice. *Nature (London)* 395, 755–756

29a. Takeuchi, E., Irizarry, M., Duff, K., Saido, T., HsiaoAshe, K., Hasegawa, M., Mann, D., Hyman, B. and Iwatsubo, T. (2000) Lack of neuronal loss associated with age-related Aβ deposition in the neocortices of transgenic mice overexpressing both Alzheimer mutant presenilin 1 and amyloid precursor protein. *Am. J. Pathol.* 157, 331–339

30. Wong, T.P., Debeir, T., Duff, K. and Cuello, A.C. (1999) Reorganization of cholinergic terminals in the cerebral cortex and hippocampus in transgenic mice carrying mutated presenilin-1 and amyloid precursor protein transgenes. *J. Neurosci.* 19, 2706–2716
31. Dodart, J., Meziane, H., Mathis, C., Bales, K., Paul, S. and Ungerer, A. (1997) Memory and learning impairment precede amyloid deposition in the V717F PDAPP transgenic mouse. Abs. 636.5, Society for Neurosciences, New Orleans
32. Justice, A. and Motter, R. (1997) Behavioral characterisation of PDAPP transgenic Alzheimer mice. Abs 636.6, Society for Neurosciences, New Orleans
33. Chapman, P.F., White, G.L., Jones, M.W., Cooper-Blacketer, D., Marshall, V.J., Irizarry, M., Younkin, L., Good, M.A., Bliss, T.V., Hyman, B.T., Younkin, S.G. and Hsiao, K.K. (1999). Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nat. Neurosci.* 2, 271–276
34. Chen, K.S., Masliah, E., Grajeda, H., Guido, T., Huang, J., Khan, K., Motter, R., Soriano, F. and Games, D. (1998) Neurodegenerative Alzheimer-like pathology in PDAPP 717V--}F transgenic mice. *Prog. Brain Res.* 117, 327–334
35. Shirazi, S.K. and Wood, J.G. (1993) The protein tyrosine kinase, fyn, in Alzheimer's disease pathology. *NeuroReport* 4, 435–437
36. Lee, G., Newman, S.T., Gard, D.L., Band, H. and Panchamoorthy, G. (1998) Tau interacts with src-family non-receptor tyrosine kinases. *J. Cell Sci.* 111, 3167–3177
37. Cobb, B.S., Schaller, M.D., Leu, T.H. and Parsons, J.T. (1994) Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. *Mol. Cell Biol.* 14, 147–155
38. Zhang, C., Qiu, H.E., Krafft, G.A. and Klein, W.L. (1996) A beta peptide enhances focal adhesion kinase/Fyn association in a rat CNS nerve cell line. *Neurosci. Lett.* 211, 187–190
39. Geula, C., Wu, C.K., Saroff, D., Lorenzo, A., Yuan, M. and Yankner, B.A. (1998) Aging renders the brain vulnerable to amyloid β-protein neurotoxicity. *Nat. Med. (N.Y.)* 4, 827–828
40. Duff, K., Knight, H., Refolo, L.M., Sanders, S., Yu, X., Picciano, M., Malester, B., Hutton, M., Adamson, J., Goedert, M., et al. (2000) Characterization of pathology in transgenic mice over-expressing human genomic and cDNA tau transgenes. *Neurobiol. Dis.* 7, 87–98
41. Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., et al. (1998) Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature (London)* 393, 702–705
42. Goedert, M., Spillantini, M.G., Crowther, R.A., Chen, S.G., Parchi, P., Tabaton, M., Lanska, D.J., Markesbery, W.R., Wilhelmsen, K.C., Dickson, D.W., et al. (1999) Tau gene mutation in familial progressive subcortical gliosis. *Nat. Med. (N.Y.)* 5, 454–457
43. Spillantini, M.G., Murrell, J.R., Goedert, M., Farlow, M.R., Klug, A. and Ghetti, B. (1998) Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7737–7741

EXHIBIT H

Review

Mammalian Genome

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Trans-NIH neuroscience initiatives on mouse phenotyping and mutagenesis

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Abstract. In the post-genomic era, the laboratory mouse will excel as a premier mammalian system to study normal and disordered biological processes, in part because of low cost, but largely because of the rich opportunities that exist for exploiting genetic tools and technologies in the mouse to systematically determine mammalian gene function. Many robust models of human disease may therefore be developed, and these in turn will provide critical clues to understanding gene function. The full potential of the mouse for understanding many of the neural and behavioral phenotypes of relevance to neuroscientists has yet to be realized. With the full anatomy of the mouse genome at hand, researchers for the first time will be able to move beyond traditional gene-by-gene approaches and take a global view of gene expression patterns crucial for neurobiological processes. In response to an action plan for mouse genomics developed on the basis of recommendations from the scientific community, seven institutes of the National Institutes of Health (NIH) initiated in 1999 a mouse genetics research program that specifically focused on neurobiology and complex behavior. The specific goals of these neuroscience initiatives are to develop high-throughput phenotyping assays and to initiate genome-wide mutagenesis projects to identify hundreds of mutant strains with heritable abnormalities of high relevance to neuroscientists. Assays and mutants generated in these efforts will be made widely available to the scientific community, and such resources will provide neuroscientists unprecedented opportunities to elucidate the molecular mechanisms of neural function and complex behavior. Such research tools ultimately will permit the manipulation and analysis of the mouse genome, as a means of gaining insight into the genetic bases of the mammalian nervous system and its complex disorders.

Introduction

Genetic factors contribute to virtually every human disease by conferring susceptibility or resistance, affecting the severity or progression of disease, and interacting with environmental factors that modify disease course and expression. In the post-genomic age, the laboratory mouse undoubtedly will play a pivotal role in understanding the function of mammalian genes. Major technological advances in the last decade have been developed that enable researchers to manipulate the mouse genome in a highly targeted and predictable way. Such advances include transgenics (Palmiter and Brinster 1986), capitalizati n on the pluripotency and germline potential of embryonic stem (ES) cells (Capecchi 1989), gene-targeting (Joyner 1993), and the discovery of the highly potent chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) (Russell et al. 1979; Hitotsumachi et al. 1985; Rinchik 1991; Shedlovsky et al. 1993; Schimenti and Bucan 1998; Brown and Nolan 1998; Hrabe de Angelis and Balling 1998). Such technologies, in combination with a draft version of the mouse genome sequence expected in 2001, will provide researchers with unprecedented opportunities for global analyses of mammalian gene and protein function.

Studies in the mouse have already provided important insights into our understanding of genes and pathways involved in a variety of human diseases (Bedell et al. 1997a, 1997b). Given the conservation of cellular and developmental processes from mouse to humans, an important approach to studying the genetic basis of human disease is to map and characterize genes influencing related biological processes in the mouse. Isolating human homologs of newly identified genes in a mouse pathway can in turn elucidate the corresponding human pathway.

Targeted or 'gene-driven' strategies have played a central role in the discovery of complex molecular pathways in the mouse and other model systems. While this approach is successful, it does not permit identification of large numbers of mutations across the genome in genes whose function remains in part or wholly unknown. Gene targeting and other directed strategies, e.g., generating gene-trap insertional mutations in embryonic stem cells (Brennan and Skarnes 1999; Wiles et al. 2000), may now be applied on a large scale (Leighton et al. 2001). To date, fewer than 5,000 mutations among approximately 30,000 mammalian genes (International Human Genome Sequencing Consortium 2001; Venter et al. 2001) have been identified in the mouse through gene-driven approaches (Nadeau et al. 2001).

Despite the existence of thousands of mouse mutations in a number of genes, the functions of the remaining genes have not been characterized, and the genetic bases of neural function and complex behavior are still poorly understood. The remaining challenge, termed the 'phenotype gap' (Brown and Peters 1996), is obtaining mutants for these remaining genes and characterizing phenotypes of these mutants. An extremely powerful and useful strategy that complements the gene-driven is a 'phenotype-driven' or 'forward genetic' approach, in which random mutagenesis across the genome is utilized to identify novel phenotypes from which relevant genes and pathways are characterized (Takahashi et al. 1994). This strategy, which does not require a priori identification of underlying genes and pathways, has been exploited with tremendous success in *Drosophila* and other organisms through screening for specific phenotypic abnormalities or defects in the progeny of chemically mutagenized animals. ENU is highly efficient for generating such mutations and has thus been the chemical of choice for the majority of genome-wide mutagenesis efforts in the mouse (Schimenti and Bucan 1998; Hrabe de Angelis et al. 2000; Justice 2000; Nolan et al. 2000a). An important demonstra-

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tion of the utility of this approach was the discovery of the *Clock* mutation by Takahashi and colleagues (Vitaterna et al. 1994). Using a combined strategy of positional cloning and transgenic rescue, the *Clock* gene was identified at the molecular level and was shown to be a novel member of the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription factors (Antoch et al. 1997; King et al. 1997). The *Clock* gene was the first mammalian clock gene to be cloned and provided an important molecular entry point for the elucidation of the circadian clock mechanism in animals. Moreover, *Clock* represents a 'proof of principle' that forward genetics can be used in mice for discovery of genes regulating the nervous system and behavior.

In the spring of 1998, the Director, National Institutes of Health (NIH), convened a group of scientists to make recommendations to the NIH regarding priorities for generating mouse genomics and genetics resources. To implement these recommendations, a trans-NIH coordinating group was established and an action plan developed (Battey et al. 1999). Major initiatives included in the plan were the development of new high-throughput phenotyping protocols and the establishment of mouse mutagenesis and phenotyping facilities.

Seven NIH institutes that support neuroscience research [National Institute of Mental Health (NIMH), National Institute of Deafness and Other Communication Disorders (NIDCD), National Institute of Neurological Disorders and Stroke (NINDS), National Institute on Aging (NIA), National Eye Institute (NEI), National Institute on Drug Abuse (NIDA), National Institute on Alcohol Abuse & Alcoholism (NIAAA)] formed a subgroup of the trans-NIH coordinating group, in order to develop mouse genomics and genetics resources that would be of specific value to the broad neuroscience and behavioral research communities. Specific neuroscience initiatives launched in 1999 and 2000 have focused on the development of reliable high-throughput phenotyping assays, and the initiation of phenotyping and genome-wide mutagenesis projects. These and other NIH activities related to mouse genomics and genetics resources are described at http://www.nih.gov/science/models/mouse/. In this paper, we present an overview of the research currently supported under these initiatives.

New phenotyping assays and protocols

Phenotype-driven approaches to understand genes and their functions in the nervous system pose unique challenges. Behavioral neuroscientists have developed many assays to assess numerous behavioral domains, including developmental milestones, neurological reflexes, motor functions, sensory abilities, learning and memory, cognition, social behaviors, as well as rodent models relevant to fear, panic, anxiety, depression, bipolar disorder, schizophrenia, reward, and drug addiction (Crawley 2000). Efforts to date have met with modest success, and a substantial number of transgenic and knockout mice have been studied and alterations identified in learning and memory, long-term potentiation, fear conditioning, anxiety, aggression, and maternal behavior (Tarantino and Bucan 2000). One complexity in this area has been the fact that many researchers have frequently employed idiosyncratic versions of behavioral tests and protocols, leading to past failures to replicate findings in different laboratories. Recent work has shown that despite efforts at standardization, systematic behavioral differences were observed across three labs, with the magnitude of genetic differences dependent upon the specific testing lab (Crabbe et al. 1999).

The widespread availability of standardized phenotyping protocols and assays of high reliability to screen for a broad range of defects in neural function and complex behavior will have an enormous impact on neuroscience research in the mouse, and will accelerate understanding of gene function and expression. The need for reliable protocols of high sensitivity is salient, given the likelihood that many very interesting behavioral abnormalities in heterozygotes are subtle. Another critical factor is the capability of phenotyping protocols for high-throughput characterization, permitting their utilization in large-scale, genome-wide mutagenesis. Development of new phenotypic assays also will permit broader characterization of the large number of targeted mouse mutations previously identified. High-throughput, sensitive, highly reliable, and standardized protocols also will be useful for gathering baseline data from multiple inbred strains commonly used in ongoing, community-wide efforts (Paigen and Eppig 2000; Nadeau et al. 2001).

One of the recommendations made to NIH by the scientific community was to develop new phenotyping protocols to permit complete characterization of mutagenized mice (Battey et al. 1999). In January 1999, seven NIH institutes that support neuroscience research (NIMH, NIDCD, NINDS, NIA, NEI, NIDA, NIAAA) published a Request for Applications (RFA) to develop objective and standardized criteria and new, cost-effective, high-throughput phenotyping tools and methods to assess specific components of neural function and complex behavior in the mouse (http://grants.nih.gov/grants/guide/rfa-files/RFA-MH-99-006.html). A major goal of this initiative is to develop protocols, assays, assessment criteria, data on reference strains, and other material and information generated in funded projects that will be made widely available to the scientific community. Table 1 presents information on the phenotypes under study, and the assays under development, in the 19 projects funded under this initiative in 1999 and 2000.

NIH required grantees funded under this RFA to propose a plan for sharing the research resources generated through the grant, including phenotyping assays and protocols, standardized and operational criteria, training manuals, quantitative scores of reference animals on behavioral and nervous system phenotyping assays. These resources will be distributed to the scientific community through a web site that will be available in 2002.

High-throughput phenotyping and mutagenesis facilities

Background. Studies of naturally occurring polygenic variation [e.g., quantitative trait loci (QTL) mapping approaches] to identify functional pathways continue to face many challenges (Nadeau and Frankel 2000). While the discovery of spontaneous mutations has provided the genetics community with powerful insights into many basic biological processes, their usefulness is limited by low frequency and the problems of detecting subtle pleiotropic effects (Nadeau 2000). A powerful alternative strategy for functional studies that has been applied quite successfully to other organisms such as *C. elegans, Drosophila,* and zebrafish is the isolation of mutations through chemical induction. ENU is a powerful germline mutagen that causes single nucleotide mutations. The highest mutation rates occur in mouse pre-meiotic spermatogonial stem cells, with single-locus mutation frequencies of 6×10^{-3} to 1.5×10^{-3} per mutagenized genome (Hitotsumachi et al. 1985), depending on drug dose used (Justice 2000). Chemical mutagenesis by ENU has great potential to generate an abundance of mutations in phenotype-driven screens, given that each of the approximately 30,000 mammalian genes is a potential target for inducing a mutation with a demonstrable, heritable phenotype.

Large-scale ENU mutagenesis allows researchers access to more genetic variants from which to select mutants for further study, e.g., those with more striking phenotypes that are not inherently polygenic. Furthermore, the sequence of candidate genes or genomic sequence in critical regions between the mutated and parental strains can be more easily compared if the mutations are isolated on isogenic background. For these reasons, it has been suggested that gene disc very by ENU mutagenesis will be more successful than QTL approaches (e.g., Nadeau and Frankel 2000).

Table 1. New Neuroscience-Relevant Phenotyping Assays Under Development.

Phenotype	Principal Investigator	Institution	Tests Under Development
Behavior and auditory system	James Willott	Northern Illinois University	Prepulse inhibition, habituation of the acoustic startle response, fear-potentiated startle, freezing paradigms
Behavior and cognition	Michela Gallagher	Johns Hopkins University	Retention for exposure to novel olfactory/gustatory information, appetitive Pavlovian conditioning, olfactory-guided learning task, neuroanatomical lesion studies, analysis of neural mechanisms using pharmacological assessment, learning relative to hormone status in female mice
Behavior and cognition	Ilan Golani	Tel Aviv University	Exploratory behavior through an automatically measured time-series of animal location
Behavior and cognition	Richard Paylor	Baylor College of Medicine	Neurological screen, open-field, light-dark test, rotorod, prepulse inhibition, and conditioned fear
Behavior and cognition	Alcino Silva	UCLA	Fear conditioning, social recognition, neuroanatomical lesion studies
Behavior and cognition	Douglas Wahlsten	University of Alberta	Motor coordination/ataxia, learning/memory, effects of alcohol and fluoxetine on behavior
Cortical function, EEG, epilepsy	Jeffrey Noebels	Baylor College of Medicine	Surface cortical EEG, depth-recorded hippocampal EEG, visual evoked potentials, brainstem auditory evoked potentials in awake and behaving mice (including multiparametric assessment related to time of day, age, and behavioral state)
Anatomy	Daniel Goldowitz	University of Tennessee Health Science Center	Neurohistological screen to assess cytoarchitecture, myelinated fiber pathways, terminal fields, astroglia and neuronal populations, activity state, and proliferative populations in developing and aged mice
Gustatory system	John Glendinning	Barnard College	Brief-access taste testing with automated gustometer to derive a robust concentration-response function
Gustatory system	Michael Tordoff	Monell Chemical Senses Center	Fine-tuning long-term, two-bottle choice tests, optimization of the brief-exposure or "lickometer" test
Olfactory system	Burton Slotnick	American University	Simple, rapid, and reliable computer-controlled tests for odor detection, odor detection threshold, odor discrimination, odor memory, and odor quality recognition, metric atlas of mouse olfactory bulb
Olfactory system	Steven Youngentob	SUNY Health Science Center	Estimation of the threshold of stimulus-induced sniffing (i.e., respiratory changes) to rapidly quantify odorant sensitivity
Visual system	Edward Pugh	University of Pennsylvania	Electroretinogram (ERG), including production of light stimulation standards and standard values for each measurable ERG parameter, quantitative pupillometric protocols, illumination standards for rearing, electroretinography, pupillometry, and retinal histology. These assays will be used to quantify the effects of rearing illumination history and to chart the natural developmental course of the phenotyping parameters from 2 weeks to 1 year of age
Visual system	Michael Stryker	UCSF	Swept visually evoked potentials recorded over the visual cortex to define the visibility of stimuli of a range of contrasts and spatial frequencies
Visual system	Robert Williams	University of Tennessee, Memphis	Cryostat sectioning, video-enhanced DIC microscopy for eye, retinal, and optic nerve architecture, electroretinograms, pupillary reflexes, and optical measurement of eyes in both sexes and wide range of ages
Auditory system	Glen Martin	University of Miami Ear Institute	Distortion-product otoacoustic emissions as screen for cochlear abnormalities at two ages in the same mouse
Vestibular system	Sherri Jones	University of Missouri School of Medicine	Stimulation and recording hardware and software for recording vestibular evoked potentials, far-field evoked potential techniques
Epilepsy	Wayne Frankel	Jackson Laboratory	Electro- and chemo-convulsive threshold tests for screening anti-convulsants; electrostimulation paradigms for high-throughput screens related to drug response
Sleep	Craig Heller	Stanford University	Simple piezoelectric transducer comprising the flexible floor upon which the animal rests, so that distension of the floor by respiratory or other movements produces electrical signals; validation of system by comparing piezo recordings with EEG recordings

Ongoing mutagenesis screens for dominant mutations in the United Kingdom and Germany have already identified an array of novel mutants (Hrabe de Angelis et al. 2000; Nolan et al. 2000a). The Munich-based German project focuses on postnatal abnormalities comprising congenital malformations and biochemical, hematological, and immunological defects (Soewarto et al. 2000), while the Harwell-based United Kingdom effort focuses on sensory, neurological, and neuromuscular phenotypes assessed using the SHIRPA protocol (Rogers et al. 1997) and behavioral assays that measure locomotor activity and pre-pulse inhibition of the acoustic startle response (Nolan et al. 2000b). Other mutagenesis centers are being established in Japan, Canada, and Australia. The focus of the comprehensive but broad phenotypic screens in each of these projects is n t on neural function and behavior.

Another recommendati n made to NIH by the scientific community was to support large-scale mutagenesis facilities, in order to accelerate functional analyses of mouse biology and to generate critical insights into the molecular mechanisms controlling normal and disrupted bi logical processes (Battey et al. 1999). In March 1999, seven NIH institutes that support neuroscience research (NIMH, NIDCD, NINDS, NIA, NEI, NIDA, NIAAA) published an RFA to establish facilities for large-scale mutagenesis and phenotyping of neural function and complex behavior in the mouse (http://grants.nih.gov/grants/ guide/rfa-files/RFA-MH-99-007.html). This initiative, by focusing intensively on one (albeit) broad domain of phenotypes, was designed to be complementary to other large-scale worldwide mutagenesis efforts. Neuroscience-focused mutagenesis and phenotyping facilities established by this RFA are expected to serve as a national resource by producing mutant strains that will be made widely available to the scientific community.

Three facilities were funded in 2000 and 2001 under this RFA to conduct large-scale mutagenesis and screen for neuroscience-relevant mutations. An overview and descriptive information are provided in Table 2. Greater details regarding mutagenesis protocols and study designs will be presented in subsequent *Mammalian Genome* papers by the principal investigators.

Experimental design. The three facilities intend to generate large numbers of ENU-mutagenized mice and screen for genome-wide recessive (primarily), dominant, or semi-dominant mutations by

Table 2. Three large-scale neuroscience-focused mutagenesis projects.

	Neuroscience Mutagenesis Facility	Northwestern University Neurogenomics Project	Tennessee Mouse Genome Consortium
Principal Investigator	Wayne Frankel	Joseph Takahashi	Daniel Goldowitz
Recipient Institution	Jackson Laboratory	Northwestern University	University of Tennessee Health Science Center
Additional Sites	Monell Chemical Senses Center University of Pennsylvania University of Vermont	Columbia University Duke University University of Iowa	Oak Ridge National Laboratory Vanderbilt University Meharry Medical College University of Tennessee–Knoxville St. Jude Children's Research Hospital University of Memphis
Key Personnel	Susan Ackerman Roderick Bronson Carol Bult Gary Churchill Gregory Cox Muriel Davisson Janan Eppig Simon John Kenneth Johnson Verity Ann Letts Larry Mobraaten Juergen Naggert Patsy Nishina Timothy O'Brien John Schimenti John Sundberg Qing Yin Zheng Alexander Bachmanov Charles Wysocki Maja Bucan Wade Berrettini Robert Lenox William Falls	Lawrence Pinto Fred Turek Jon Levine Eva Redei Warren Kibbe Martha Vitaterna Sandra Siepka Kazuhiro Shimomura Eric Kandel Eleanor Simpson Marc Caron Raul Gainetdinov Val Sheffield Edwin Stone Gregory Hageman	Michael Ferkin Kristin Hamre Dabney Johnson Doug Matthews Guy Mittleman Tom Montine Eugene Rinchik Jay Snoddy Robert Williams Twum Ansah Erich Baker Randy Blakely Mike McDonald Jerry Wolff Andrea Elberger Karen Goss Monica Jablonski Mike Paulus Richard Smeyne Robert Waters Ron Wiley Danny Winder Jian Zuo
Approach	Genome-wide	Genome-wide	Region-specific (15% of genome)
Phenotypes	Motor function, seizure threshold, hearing and vision impairments, learning and memory, taste and olfaction	Circadian rhythmicity, learning and memory (fear conditioning), visual function, neuroendocrine function (HPA, HPT axes), psychostimulant response	General screens: motor and sensory function, behavior, learning and memory, neurohistology. Specific screens: aging, alcohol response, abused drug response, visual function, social behavior
Project URL	http://www.jax.org/resources/documents/nmf/	http://genome.northwestern.edu/	http://tnmouse.org/index.html

using a three-generation pedigree breeding scheme. Mutagenesis will be performed exclusively in C57BL/6J mice at The Jackson Laboratory and Northwestern University programs; the Tennessee Mouse Genome Consortium will use animals from mixed backgrounds, derived mostly from the B6 and C3Hf/R1 strains. The Jackson Laboratory project also will implement improved mutagenesis technologies by treating ES cells with chemicals other than ENU and conducting genome-wide recessive screens in two generations. The Tennessee Mouse Genome Consortium will employ regional mutagenesis, covering regions on Chrs 10, 15, 19, and X. Specifically, mutations will be produced in ~40 cM on Chr 10, half of Chr 15 (~30 cM), and ~30 cM on X. Molecular or visible (coat color) markers will be used to track these newly induced mutations. Mutations over all of Chr 19 also will be produced, through use of a novel protocol to mutagenize consomic mice (Williams 1999). ENU-induced mutations in ~15% of the mouse genome will be screened, isolated, and characterized by the Tennessee Mouse Genome Consortium. In all three projects, high-throughput phenotyping protocols will be utilized to screen G3 mice and isolate and characterize mutations that alter targeted phenotypic domains relevant t neural function and complex behavior (see below). Other activities include: testing t establish whether detectable phenotypes are heritable; and chromosome mapping f a subset of mutants recovered from the screens to genomic regions of ~10–20 cM, in order to identify mutations that are potentially at the same locus, as well as for future positi nal cloning and gene identification studies.

Anticipated mutant yield. Each project will produce and screen between 8,000 and 10,000 third-generation (G_3) progeny per year. Over the course of the 5-year projects, a total of ~40,000–50,000 animals will be screened. At least 150–300 visible mutants with heritable phenotypes are expected to be identified per year, for a total of 750–1,500 at the end of the three projects. Approximately half of these will be mapped by the facilities to 10–20 cM genomic regions.

Specific pathogen-free (SPF) mice. Mutations were previously induced in regions on Chrs 10, 15, and X in several lines of animals at Oak Ridge National Laboratory, a conventional facility in the Tennessee Mouse Genome Consortium. As part of the current NIH-funded mutagenesis effort, all of these lines will be rederived under SPF conditions at a University of Tennessee SPF facility. Animals newly mutagenized by all three projects will be mutagenized under SPF conditions. Phenotype determination and heritability studies typically will be performed under SPF conditions.

Phenotyping screens. A key feature across facilities is the reliance on a broad range of assays to assess multiple phenotypic domains. Specific primary d mains that influence nerv us system function and complex behavior are targeted by each facility (Table 3). The Jackson Laboratory project will use a high-throughput primary screen to detect potential deviants in several neuroscience-relevant domains, including motor function, seizure threshold, hearing and

Table 3. Primary phenotypic domains and tests: neuroscience-focused mutagenesis projects.

Project	Primary Phenotypic Domain	Tests
Jackson Laboratory	Motor function	Rotorod test; grip strength
	Seizure threshold	Electroconvulsive threshold
	Hearing impairments	Acoustic startle response; prepulse inhibition fear-potentiated startle
	Vision impairments	Dysmorphology surveys; electroretinography
	Learning and memory	Fear-potentiated startle
	Taste and olfaction	Two-bottle and "buried food" test
Tennessee Mouse Genome Consortium	Abused drug response	Locomotor activity; novelty seeking; novel food neophobia
	Alcohol response	Ethanol-induced alterations in locomotor activity; two-bottle choice test
	Visual function	Eye size, structure immunohistochemistry
	Social behavior	Sexual, reproductive, and parenting behaviors; aggression/intruder test
	Aging	Morbidity and mortality, behavioral and pathological changes with aging
Northwestern University	Circadian clock function	Locomotor activity rhythms
	Learning and memory	Conditioned fear response (context-dependent and cued)
	Visual function	Electroretinography; visually evoked potentials; Fundus photography
	Neuroendocrine function	Corticosterone and TSH assay; behavioral tests (open field behavior, elevated plus maze)
	Psychostimulant response	Acute locomotor response

vision impairments, neural origins of obesity, learning and memory (as assessed by a fear-potentiated startle test), taste, and olfaction. Also included are simple, rapid qualitative screens selected from those initially developed to evaluate neurological effects of pharmacological interventions and recently re-adapted in the SHIRPA protocol for dominant mutant screen at Harwell (http://www.mgu.har.mrc.ac.uk/ mutabase/shirpa summary.html). Specific screens in this modified SHIRPA protocol to be used in The Jackson Laboratory project include transfer arousal, palpebral closure, piloerection, skin color, tail elevation, limb grasping, wire maneuver, visual placing, startle click box, pinna reflex, corneal reflex, negative geotaxis, trunk curl, and touch escape.

The Tennessee Mouse Genome Consortium project will conduct a high-throughput primary screen that includes behavioral assays, neurohistology, and imaging. Soft-tissue and skeletal morphology are assessed through whole-body imaging, and animals are screened on five phenotypic domains within neuroscience: abused drug response, alcohol response, visual function, social behavior, and aging. Mice with specific mutant phenotypes are screened by one of four secondary domains: auditory function, nociception, abused drug response, and learning and memory.

The Northwestern University project focuses on five phenotypic domains within neuroscience: circadian rhythmicity, learning and memory (fear conditioning), visual function, neuroendocrine function (hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid axes), and psychostimulant response. In addition, a set of tests that constitute a "preliminary assessment" will provide supplemental information to aid in the interpretation of primary phenotypic data. These include assessments of growth, locomotion, hearing, and stress- or anxiety-related behavior.

Governance. Each of the three mutagenesis projects is funded by NIH under a cooperative agreement, an assistance mechanism in which there is anticipated substantial NIH programmatic involvement with the principal investigators over the course of the project. Collaborative activities of the principal investigators and NIH staff will occur under the rubric of the Neuroscience Steering Committee (NSC), which will serve as the governing board of all three projects. NSC includes the three project principal investigators, NIH program staff, and three scientific advisors with relevant scientific expertise who are not affiliated with any of the facilities. Robert Karp from the National Institute on Alcohol Abuse & Alcoholism is the current NIH program staff representative, and the three scientific advisors are Richard Thompson, (University of Southern California), Stephen Brown (MRC Mammalian Genetics Unit and UK Mouse Genome Centre at Harwell), and J. Michael Cherry (Stanford University), who serves as NSC chair. NSC coordinates the activities f the mutagenesis facilities and the exchange of information and biomaterials with the wider scientific community. While mutagenesis of multiple regions of varying size across the whole mouse genome will be conducted, the NSC will also establish priorities for the study of particular phenotypes and genomic regions. Advice on the direction of research is also provided by each project's external advisory board.

Oversight of NSC is provided by the Mouse Gen mics and Genetics Scientific Panel (MSP), an NIH advisory committee. MSP ensures coordination among the three neuroscience-focused mutagenesis projects and one mutagenesis project focused on developmental defects that was funded under RFA HD-99-007 (http://grants.nih.gov/grants/ guide/rfa-files/RFA-HD-99-007. html), while evaluating their progress in relation to the evolving goals for trans-NIH initiatives on mouse genetics and genomics. MSP will consist of about 10 scientific advisors not affiliated with any of the mutagenesis and phenotyping facilities, and who are not members of NSC or the steering committee that oversees the developmental defects-focused mutagenesis facility. MSP advisors will be selected for their broad expertise in relevant topics such as developmental biology, aging, neurobiology, behavior, mutagenesis, phenotyping, mouse genetics, husbandry, genomics, and bioinformatics. Current members include Carol Barnes (University of Arizona), Aravinda Chakravarti (Johns Hopkins University), Kenneth Fasman (AstraZeneca), Nancy Jenkins (National Cancer Institute), George Koob (Scripps Research Institute), Raju Kucherlapati (Albert Einstein College of Medicine), Gail Martin (University of California, San Francisco), Miriam Meisler (University of Michigan), Luis Parada (University of Texas, Southwestern Medical Center), Philippe Soriano (Fred Hutchinson Cancer Research Center), Cori Bargmann (University of California, San Francisco), and Charles Zucker (University of California, San Diego), with additional members being added at a later date.

Availability of mutant strains to the scientific community. The sharing of materials and data in a timely manner has been an essential element in the rapid progress that has occurred in biomedical research. However, many scientists are increasingly frustrated by growing difficulties and delays in negotiating the terms of access to research tools. Excessive intellectual property restrictions can stifle the broad dissemination of new discoveries and limit future avenues of research and product development (Heller and Eisenberg 1998). Restricted availability of unique research resources can impede the advancement of research and delivery of medical care (Bobrow and Thomas 2001). Sharing of biomaterials in a timely manner, on the other hand, has been an essential element in the rapid progress that has been made in the genetic analysis of mammalian and nonmammalian genomes (e.g., Adams et al. 2000; International Human Genome Sequencing Consortium

2001). The success of the NIH neuroscience-focused mouse mutagenesis initiative depends on the timely and unencumbered availability of mutant strains to the scientific community, in order to ensure a publicly accessible set of genetically altered animals that can be used in a wide range of future biomedical research projects.

Under most circumstances, NIH recognizes the rights of grantees normally to elect and retain title to subject inventions developed with Federal funding under the provisions of the Bayh-Dole Act (Senate Committee on the Judiciary Subcommittee on Patents and Trademarks 1994). Under the cooperative agreement arrangement, if each of the recipient/subcontractor organizations retained and exercised its intellectual property rights under the Bayh-Dole Act over the generation of all mutant strains, there would be potential consequences that would not be consistent with the intent of the Bayh-Dole Act. Each of several parties would control the intellectual property over random numbers of mutant mice; this would make it more difficult to use the animals to their fullest extent to enable the development of products that can improve the public health. In that event, different mutant animals would be controlled by different sources. Any scientist interested in developing a comprehensive animal model of a given disease, which may involve use of several different strains that each express different phenotypic abnormalities, would undoubtedly have to deal with several owners and a complex set of intellectual property demands and constraints. This complication would directly undermine the fundamental purpose of the neuroscience mutagenesis initiative, i.e., to create research resources broadly accessible for the scientific community.

The Bayh-Dole Act at 35 U.S.C. § 202(a)(ii) enables the Government to restrict or eliminate the right to retain title "in exceptional circumstances when it is determined by the Agency that restriction or elimination of the [recipient/subcontractor's] right to retain title to any subject invention will better promote the policy and objectives of [the Bayh-Dole Act]." The NIH Director has made a determination that such exceptional circumstances exist and justify the restriction or elimination of patent rights of the organizations and their subcontractors funded to establish neuroscience- and developmental defects-focused mutagenesis facilities, in order to assure that the policy and objectives of 35 U.S.C. § 200 are better promoted. Such a Demonstration of Exception Circumstances (DEC) ensures that the Government protects the public and the research community against nonuse or unreasonable use of such inventions by making the mutant strains generated from the NIH mutagenesis initiatives rapidly and freely accessible to the scientific community for further research and development, in the expectation that unfettered access will more rapidly and effectively lead to products of benefit to the public.

Consequently, it is a requirement that the three neuroscience-focused mutagenesis projects funded under RFA MH-99-007 (and the one mutagenesis project focused on developmental defects that was funded under RFA HD-99-007) make the results and accomplishments of their activities available to the scientific community as a national resource. Mutant strains (including sperm and embryos), as well as phenotypic and genetic mapping data, will be obtained through a website that will become available in 2002. These different resources will provide new and critical insights into the molecular mechanisms governing normal and disrupted neurobiological processes, and their wide availability is expected to lead to rapid advances in biomedical science that are necessary for understanding the molecular basis of human disease.

Coordination of mouse neuroscience initiatives

The initiative to develop new phenotyping assays was started prior to the mutagenesis initiative, in order to enable development of high-throughput phenotyping assays that could be utilized in large-scale mutagenesis efforts. NIH is working to encourage investigators supported under these efforts to collaborate, thereby permitting screening of mutant strains on a broader range of phenotypes relevant to neuroscience. One option for collaboration is for investigators to visit the mutagenesis facilities and utilize their assays and protocols for screening. Each of the mutagenesis projects has a plan to permit guest investigators not associated with the facility to make use of the facility to screen for, and/or to examine, alterations in neural function and behavior not otherwise being studied at the facility. This option may provide investigators funded under the neuroscience phenotyping initiative the opportunity to collaborate with the ongoing mutagenesis projects and utilize their new high-throughput assays to screen for novel mutations. Additional phenotype analysis of interesting mouse strains will further increase the scientific value of these mutant strains to the scientific community and accelerate identification of new models of neurobehavioral disorders. NSC is also exploring the possibility of making mutagenized mice not displaying phenotypes of immediate interest to investigators in the mutagenesis facilities available to investigators wishing to screen them for additional phenotypes.

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References

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD et al. (2000) The genome sequence of *Drosophila melanogaster*. Science 287, 2185–2195

Antoch MP, Song EJ, Chang AM, Vitaterna MH, Zhao Y et al. (1997) Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. Cell 89, 655–667

Battey J, Jordan E, Cox D, Dove W (1999) An action plan for mouse genomics. Nat Genet 21, 73–75

Bedell MA, Jenkins NA, Copeland NG (1997a) Mouse models of human disease. Part I: techniques and resources for genetic analysis in mice. Genes Dev 11, 1–10

Bedell MA, Largaespada DA, Jenkins NA, Copeland NG (1997b) Mouse models of human disease. Part II: Recent progress and future directions. Genes Dev 11, 11–43

Bobrow M, Thomas S (2001) Patents in a genetic age. Nature 409, 763–764

Brennan J, Skarnes WC (1999) Gene trapping in mouse embryonic stem cells. Methods Mol Biol 97, 123–138

Brown SD, Nolan PM (1998) Mouse mutagenesis—systematic studies of mammalian gene function. Hum Mol Genet 7, 1627–1633

Brown SD, Peters J (1996) Combining mutagenesis and genomics in the mouse—closing the phenotype gap. Trends Genet 12, 433–435

Capecchi MR (1989) Altering the genome by homologous recombination. Science 244, 1288–1292

Crabbe JC, Wahlsten D, Dudek BC (1999) Genetics of mouse behavior: interactions with laboratory environment. Science 284, 1670–1672

Crawley JN (2000) *What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice.* (New York, NY: Wiley-Liss)

Heller MA, Eisenberg RS (1998) Can patents deter innovation? The anticommons in biomedical research. Science 280, 698–701

Hitotsumachi S, Carpenter DA, Russell WL (1985) Dose-repetition increases the mutagenic effectiveness of N-ethyl-N-nitrosourea in mouse spermatogonia. Proc Natl Acad Sci USA 82, 6619–6621

Hrabe de Angelis MH, Balling R (1998) Large scale ENU screens in the mouse: genetics meets genomics. Mutat Res 400, 25–32

Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D et al. (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet 25, 444–447

International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. Nature 409, 860–921

Joyner AL (1993) *Gene Targeting: A Practical Approach.* (New York, NY: Oxford University Press)

Justice MJ (2000) Capitalizing on large-scale mouse mutagenesis screens. Nat Rev Genet 1, 109–115

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M et al. (1997) Positional cloning of the mouse circadian clock gene. Cell 89, 641–653

Leighton PA, Mitchell KJ, Goodrich LV, Lu X, Pinson K et al. (2001) Defining brain wiring patterns and mechanisms through gene trapping in mice. Nature 410, 174–179

Nadeau JH (2000) Muta-genetics or muta-genomics: the feasibility of large-scale mutagenesis and phenotyping programs. Mamm Genome 11, 603–607

Nadeau JH, Frankel WN (2000) The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. Nat Genet 25, 381–384

Nadeau JH, Balling R, Barsh G, Beier D, Brown SD et al. (2001) Sequence interpretation. Functional annotation of mouse genome sequences. Science 291, 1251–1255

Nolan PM, Peters J, Strivens M, Rogers D, Hagan J et al. (2000a) A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat Genet 25, 440–443

Nolan PM, Peters J, Vizor L, Strivens M, Washbourne R et al. (2000b) Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource. Mamm Genome 11, 500–506

Paigen K, Eppig JT (2000) A mouse phenome project. Mamm Genome 11, 715–717

Palmiter RD, Brinster RL (1986) Germ-line transformation of mice. Annu Rev Genet 20, 465–499

Rinchik EM (1991) Chemical mutagenesis and fine-structure functional analysis of the mouse genome. Trends Genet 7, 15–21

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ et al. (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome 8, 711–713

Russell WL, Kelly EM, Hunsicker PR, Bangham JW, Maddux SC et al. (1979) Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. Proc Natl Acad Sci USA 76, 5818–5819

Schimenti J, Bucan M (1998) Functional genomics in the mouse: phenotype-based mutagenesis screens. Genome Res 8, 698–710

Senate Committee on the Judiciary Subcommittee on Patents and Trademarks. The Bayh-Dole Act, A Review of Patent Issues in Federally Funded Research: Hearings on Pub. L. No. 96-517, 103 Congress, 2nd session, 1–2. 1994

Shedlovsky A, McDonald JD, Symula D, Dove WF (1993) Mouse models of human phenylketonuria. Genetics 134, 1205–1210

Soewarto D, Fella C, Teubner A, Rathkolb B, Pargent W et al. (2000) The large-scale Munich ENU-mouse-mutagenesis screen. Mamm Genome 11, 507–510

Takahashi JS, Pinto LH, Vitaterna MH (1994) Forward and reverse genetic approaches to behavior in the mouse. Science 264, 1724–1733

Tarantino LM, Bucan M (2000) Dissection of behavior and psychiatric disorders using the mouse as a model. Hum Mol Genet 9, 953–965

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ et al. (2001) The sequence of the human genome. Science 291, 1304–1351

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL et al. (1994) Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 264, 719–725

Wiles MV, Vauti F, Otte J, Fuchtbauer EM, Ruiz P et al. (2000) Establishment of a gene-trap sequence tag library to generate mutant mice from embryonic stem cells. Nat Genet 24, 13–14

Williams RW (1999) A targeted screen to detect recessive mutations that have quantitative effects. Mamm Genome 10, 734–738